AD

ANNUAL PROGRESS REPORT

1 July 1964-30 June 1965
(FY 1965)

Edited by: Colonel Lyman P. Frick, MSC, Director

30 September 1965

U. S. ARMY TROPICAL RESEARCH MEDICAL LABORATORY
APO New York 09851

AD

ANNUAL PROGRESS REPORT, FY 1965

Reports Control Symbol: RCS-MEDDH-288

Edited by: Colonel Lyman P. Frick, MSC, Director

30 September 1965

Fiscal Year 1965 Projects:

3A014501A71F - Military Internal Medicine

3A014501B71P - Basic Research in Support of Military Medicine

3A014501A71Q - Communicable Diseases and Immunology

3A013001A91C - In-House Laboratory Independent Research

U. S. ARMY TROPICAL RESEARCH MEDICAL LABORATORY
APO New York 09851

SUMMARY

The research program of the U. S. Army Tropical Research Medical Laboratory is devoted to the study of tropical diseases of military importance.

The research projects described herein are related to investigations of Schistosomiasis, Tropical Sprue and Common Diarrheas.

FOREWORD

The FY 1965 Annual Progress Report is a general review of the following research projects conducted by the U. S. Army Tropical Research Medical Laboratory:

3A014501A71F - Military Internal Medicine

3A014501B71P - Basic Research in Support of Military Medicine

3A014501A71Q - Communicable Diseases and Immunology

3A013001A91C - In-House Laboratory Independent Research

The above projects are divided into tasks and work units under which research studies are performed:

3A014501A71F-02 - Metabolism and Nutrition

- -36 Hematology
- -37 Gastrointestinal Function and Histology
- -38 Metabolic Changes in Malnutrition

3A014501B71P-11 - Zoological Sciences

- -01 Biology of Schistosomiasis and Other Parasitoses
- -02 Ecology and Control of Intermediate Hosts of Schistosomiasis and Other Parasitoses

3A014501A71Q-01 - Communicable Diseases

- -06 Diarrheal Diseases
- -07 Parasitic Diseases

3A013001A91C - In-House Laboratory Independent Research

- -1 Study of Human Chromosomes in the Tropics
- -2 The Ultramicroscopic Structure of the Intestinal Mucosa in Tropical Sprue
- -3 Immunologic Significance of Cross-Protection in Members of Group A Arboviruses

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Reference to commercial products used in conducting research is not intended to mean indorsement of the products.

In conducting research described in this report, the investigators adhered to the principles of laboratory animal care as established by the National Society for Medical Research.

ACKNOWLEDGMENT

The research reported herein has been greatly facilitated by the enthusiastic cooperation and assistance rendered by many of the professional and administrative staffs of the School of Medicine, School of Tropical Medicine, University of Puerto Rico.

Special acknowlegment is made of the generous assistance given by Adan Nigaglioni, MD, Dean, School of Medicine, and Mario Garcia-Palmieri, MD, Professor and Head, Department of Medicine, University of Puerto Rico School of Medicine.

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RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYM
		DA 0A6404	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT	6 SECURITY WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
1 07 65	A. New	U	U	"NA"	NL	A. Work Unit

10a CURRENT NUMBER/CODE: 61145011 3A014501B71R 02 070

10b PRIOR NUMBER CODE: 61145011 3A014501B71F 02 36

11 TITLE: (U) Hematology (PR)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical Medicine	01 63	"NA"	Other | DA

16 PROCURE METHOD	17 CONTRACT GRANT		18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
	a NUMBER	b DATE			
C	"NA"		PRIOR FY 65	1	35
	c TYPE	d AMOUNT	CURRENT FY 66	1	50

19 GOV'T LAB INSTALLATION ACTIVITY

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21 TECHNOLOGY UTILIZATION	22 COORDINATION
"NA"	"NA"

23 KEYWORDS: Megaloblastic anemia, Erythroid maturation, Tropical sprue, Folic Acid, Vitamin B_{12}

24 (U) Tech Objective - Various aspects of megaloblastic anemia are being studied. The relationship of folic acid and Vitamin B_{12} in erythroid maturation is under investigation. It is anticipated that further insight into this relationship will result.

25 (U) Approach - Microbiological assays for Vitamin B_{12} and folic acid are run on serum and whole blood obtained from patients with megaloblastic anemia associated either with tropical sprue or nutritional deficiency. The patients are maintained on a controlled diet low in folic acid and Vitamin B_{12} while small amounts of one or both of these substances are given by injection. Serial blood counts, bone marrow aspirations and microbiological assays are performed.

The major technical problem involves the proper performance of the microbiological assays.

26 (U) Progress - In this period 10 patients have been studied. The results show that in tropical sprue Vitamin B_{12} deficiency can be more limiting to erythropoiesis than folic acid deficiency. This fact makes difficult the evaluation of the effect of small doses (100γ or less) of folic acid.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
[ ] a COMSEC OR COMSEC RELATED [X] b NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
"NA"	"NA"

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) (FY)	36

DD FORM 1 AUG 64 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A014501B71F: Research in Biomedical Sciences

Task No. 3A014501B71F 02: Internal Medicine

Work Unit No. B71F 02 36: Hematology (PR)

Reporting Installation: U. S. Army Tropical Research Medical Laboratory
APO New York 09851

Division: Department of Medicine

Period Covered by Report: 1 July 1964 - 30 June 1965

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Report Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

BODY OF REPORT

Project No. 3A014501B71F: Research in Biomedical Sciences

Task No. 3A014501B71F C2: Internal Medicine

Work Unit No. B71F 02 36: Hematology (PR)

Study 1. Absorption studies in megaloblastic anemia in pregnancy.

Progress:

Seventy pregnant patients with a hemoglobin of 8.5 gms or less were studied. They were grouped according to the diagnosis in 3 groups: Group I was formed by 18 patients with megaloblastic anemia; Group II was composed of 25 patients with evidence of megaloblastic and iron deficiency anemia; and Group III was formed by 25 patients with iron deficiency anemia.

Two additional groups of patients with hemoglobin of 12 gms or more were studied as controls: Group IV was formed by 10 females with uncomplicated pregnancies; and Group V formed by 10 nonpregnant females less than 45 yrs. old with no medical complications.

D xylose tolerance test (5 gm dose), Vitamin A tolerance curve, serum carotene, and fecal fat collection for 3 consecutive days were done in every patient, and peroral jejunal biopsies were taken from most of them.

The patients of Group I (cases of megaloblastic anemia) showed a jejunal mucosa compatible with intestinal malabsorption in each of 12 cases biopsied. Seventeen of 18 patients had at least one abnormal absorption study, and 13 of them had at least 2 abnormal absorption tests.

Group II (those with features of megaloblastic and iron deficiency anemia) showed abnormal biopsies in 13 of 16 patients tested. Seventeen of the 23 patients (74%) who had all absorption tests done showed at least 1 abnormal test and 10 of them (43%) had at least 2 abnormal absorption tests.

Group III (those with iron deficiency anemia) had abnormal jejunal biopsies in 12 of 17 patients tested. Thirteen of 22 patients (60%) who had the absorption tests done, had at least 1 abnormal test and 5 of them had 2 abnormal tests (23%).

Group IV (pregnant women with hemoglobin of 12 gms or more) and those of Group V (nonpregnant females with normal hemoglobin) had normal biopsies and normal absorption tests in every case.

Summary:

Megaloblastic anemia of pregnancy in Puerto Rico is commonly associated with intestinal malabsorption.

Study 2. The folic acid co-factor forms in plasma and whole blood in tropical sprue patients.

Progress:

1. No significant deviation from normal was found in plasma folate activity by S. fecalis assay in tropical sprue. Whole blood folate activity when adjusted to a normal hematocrit was likewise virtually the same in anemic sprue patients as in normal individuals.

2. Whole blood folate activity for L. casei, when similarly compared was reduced in anemic sprue patients to less than half the normal value. If no correction was made for anemia, the difference was even more striking.

3. Plasma levels of L. casei activity were within the normal range in 5 of 12 subjects with the megaloblastic anemia of tropical sprue. Corrected whole blood values for L. casei activity on the other hand were below the normal limit in each of the fourteen similar subjects. For these reasons the L. casei assay of folate activity in whole blood appears to be superior to the assay of plasma in the diagnosis of tropical sprue.

4. Plasma L. casei activity was normal in 3 of 9 sprue patients in another group each of whom had marked reduction in serum Vitamin B-12 according to Euglena gracilis assays, but whole blood folate activity was low in every subject. No elevated values in plasma or whole blood folate occurred in this group of Vitamin B-12 deficient patients. Tropical sprue seems to be characterized by simultaneous reduction in both serum Vitamin B-12 and whole blood L. casei activity.

5. Because of the differential growth requirements of L. casei and S. fecalis, the results are interpreted to mean that unsubstituted folates and formyl folates are normal in both the red cells and plasma of patients with untreated tropical sprue. On the other hand, methylated folate derivatives in whole blood are significantly depressed. It is postulated that in tropical sprue an abnormality may exist in the conversion of formyl folates to methylated folate coenzyme forms.

Summary:

Evaluation of plasma and whole blood folate activity revealed that methylated folate derivaties are decreased in blood whereas unsubstituted and formyl folates are normal

Study 3. A morphological comparison of peripheral blood, bone marrow, and jejunal crypt cells in tropical sprue.

Progress:

Bone marrow and jejunal crypts are both actively proliferating systems dependent on, among other nutriments, folic acid and Vitamin B_{12}. Cellular maturation in these systems is drastically altered in tropical sprue. These alterations may be a reflection of a deficiency of folic acid and, or, Vitamin B_{12}, or of some type of antagonistic action by an unknown agent or process. By comparing the morphology of these systems in acute and chronic sprue, and by correlating these findings with studies of folic acid metabolism (serum L. casei assay, plasma folate clearance) some insight into the mechanisms of sprue may result. Thus far, excellent correlation has been found in acute sprue between bone marrow changes suggesting folic acid deficiency and enlarged jejunal crypt cells. Although the correlation does not hold as well in cases of very early sprue, treated sprue, and megaloblastic anemia due to causes other than sprue, these discrepancies suggest that the lesion of sprue begins at the intestinal level and that the bone marrow changes are secondary to subsequent deficiency.

Summary:

Excellent correlation was found of degree of cellular changes in acute sprue was found at the three sites. The sprue lesion may occur first in the intestine.

Study 4. Dietary folate deprivation in tropical sprue.

Progress:

To evaluate the importance of folic acid deficiency in the pathogenesis of tropical sprue, 6 patients with classical sprue were studied while consuming a diet containing 20 ug folate activity (S. fecalis) for 2 to 4 months. At intervals, parenteral B_{12} (1-3 ug) or folic acid (100 ug) was given. Serial studies included blood counts, serum B_{12} and folate (L. casei) assays, bone marrow, absorptive tests including fat excretion, and jejunal biopsies. Results indicate: 1. Folate deprivation aggravated the megaloblastosis, but had little effect on clinical status or absorption. Jejunal mucosal changes were observed but were more striking with dissecting microscope examination than in histologic sections. 2. Despite extremely low serum folate levels and folate deprivation, definite but partial hematologic response followed B_{12} injections (1-3 ug). 3. With low serum B_{12} levels, 100 ug folate (I.M.) was ineffective hematologically and produced minimal, if any, elevation in serum folate. Thus, in tropical sprue B_{12} deficiency can be more limiting to erythropoiesis than folate deficiency. 4. Despite extremely low serum folate levels and low folate diet, tetracycline decreased steatorrhea significantly. Although dramatic improvement with folic acid therapy is a prominent feature of tropical sprue, and although relapse frequently occurs if this

therapy is stopped, the present findings indicated that severity of the illness was not increased with increasing folate deficiency under controlled conditions.

Summary:

The clinical severity of the disease was not increased by increasing the folate deficiency.

Study 5. Site of iron absorption in human subjects.

Progress:

Little or no data are available regarding the site of most efficient iron absorption in humans. Work in animals shows the duodenum to be far more efficient than the rest of the gastrointestinal tract. This has been investigated in humans by the use of a double-isotope technique. Iron 55 and iron 59 were given with the same amount of carrier ferrous iron, on alternate days, for a total of six days. One isotope is given orally and the other by an indwelling tube placed so as to bypass the area of gastrointestinal tract under study. Two weeks after the last dose of iron, a sample of red blood cells is digested and counted to determine separately the radioactivity of each isotope. The results indicate that bypassing the first portion of the intestine to the ligament of Treitz does not significantly decrease iron absorption in normals or iron deficient subjects. In normal subjects when the tube is placed into the proximal jejunum or into the mid-lower jejunum a reduction of from 11 to 69 per cent in iron absorption occurs. In iron deficient subjects when the tube is placed into the middle to lower jejunum iron absorption was decreased from 23 to 95 per cent compared to the orally administered dose. Thus it has been shown that the upper gastrointestinal tract is more efficient for iron absorption that the lower.

Summary:

The use of a double-isotope technique has shown the upper gastrointestinal tract of man to absorb iron more efficiently than the lower.

Study 6. Effect of splenectomy and subsequent feeding of raw spleen on the leukocyte and platelet counts in the albino rat.

Progress:

It has been proved by Palmer that after splenectomy, rats develop lasting thrombocytosis and leukocytosis of moderate degree.

Recently, Beiliner reported a case of thrombophlebitic splenomegaly followed by splenectomy that 2½ years after surgery showed constant hyperthrombocytosis, neutrophilic leukocytosis with a leftward shift, monocytosis

and eosinophilia. These were seen to increase with intercurrent infection. These symptoms had been present for 2 years when nasal hemorrhages started. After treatment with raw spleen, the platelet count became normal and the nasal hemorrhages ceased; the leukocyte count was unchanged. The success in this treatment led Beiliner to believe that a deficiency of a splenic factor affecting thrombocytopoiesis may have some role in the development of the disease.

Following this line of thinking, an experiment has been set up to determine the effect of splenectomy with subsequent feeding of raw spleen on the total leukocyte and platelet counts in the pathogen free albino rat. This experiment was originated at Walter Reed Army Institute of Research, Washington, D. C. Approximately 64 pathogen free albino rats of the WRCF strain, weighing between 150-200 gm were used. A splenectomized, a control operated and an unoperated group was studied. All rats were kept on regular diet and the ones splenectomized were started on the spleen diet one week post-operatively, except the control ones which were kept some on regular diet, and some on a raw or cooked tripe diet. Total leukocyte and platelet counts, hematocrit and hemoglobin determinations were done weekly.

As the experiment progressed, the control rats were fed successively raw tripe, liver and kidney, after being returned to regular diet in alternate periods in between the change of diet. Only the rats fed raw spleen showed a decrease in platelet and leukocyte levels. This was maintained as long as the raw spleen diet was kept. A change to regular diet brought the counts close to the original post-splenectomy levels, and after being returned to the raw spleen diet again, both the platelet and leukocyte levels were lowered. In the splenectomized group, both the platelet and WBC levels dropped below normal, the platelet decrease being considerably greater than the leukocyte decrease.

In the control unoperated group, there was also a drop in both leukocyte and platelet levels, when fed raw spleen, but this drop never went below normal. The cooked spleen failed to produce identical results.

This study has been carried at the U. S. Army Tropical Research Medical Laboratory, but on a different strain of albino rats and with varying climactic conditions. The spleen feedings were altered by feeding some rats raw spleen frozen at the very moment it was obtained from the animal (in our case, beef spleen) and some others being fed raw spleen allowed to remain at room temperature for several hours, before it was frozen.

Two groups of rats were set up: Group I was kept in an environment to simulate as closely as possible the usual that exists in San Juan; Group II was kept in an air conditioned environment. The environmental conditions of Group I changed so much during the experiment, that the results obtained were erratic and not interpretable. Group II showed stable normal leukocyte and platelet levels prior to splenectomy. Post-splenectomy and while they were fed raw spleen (either ready frozen or

allowed to remain at room temperature before freezing) they showed a tendency towards a rise in both levels that was considered significant. Future plans include doing a larger set of rats along these same conditions.

Furthermore, during the course of this year two patients have been studied to see the effect of raw spleen feedings on the leukocyte and platelet levels. Patient I was a well studied case of polycythemia rubra vera and Patient II was considered normal. Patient I was fed 200 gms raw spleen diluted in 1000 cc's tomato juice daily for three weeks. Patient II was fed increasing daily amounts of raw spleen (starting on 200 gms, up to 1000 gms diluted in 1000 cc's tomato juice) for three weeks and then changed to 1000 gms splenic connective tissue diluted in 1000 cc's tomato juice for another week. Both patients failed to show any change in their platelet or leukocyte levels.

Plans are to continue studying several other patients along this line whenever they are available.

Summary:

No consistent decrease in platelets or WBC's was found in splenectomized rats in San Juan which were fed raw spleen.

Publication:

1. Effect of transferrin saturation on iron absorption in man. Munsey S. Wheby, and Genoveva Umpierre. New England Journal of Medicine 271: 1391, 1964.

RESEARCH AND TECHNOLOGY RESUME		2. GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 6405	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT	6 SECURITY WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
7 07 65	A. New	U	U	"NA"	NL	A. Work Unit

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER/CODE
61145011 3A014501B71R 02 071	61145011 3A014501B71F 02 37

11 TITLE

(U) Gastrointestinal Function and Histology (PR)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical Medicine	01 63	"NA"	Other DA

16 PROCURE METHOD	17 CONTRACT/GRANT		18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
	a DATE				
C	b NUMBER "NA"		PRIOR FY 65	2	65
	c TYPE	d AMOUNT	CURRENT FY 66	2	95

19 GOV'T LAB INSTALLATION/ACTIVITY	20 PERFORMING ORGANIZATION
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21 TECHNOLOGY UTILIZATION	22 COORDINATION
"NA"	"NA"

23 KEYWORDS Gastrointestinal, Tropical Sprue, Anemia, Xylose Tolerance, Vitamin A Tolerance, Radioactive B_{12}, Iron Absorption, Mucosal Biopsies

24 (U) Tech Objective - Gastrointestinal function and histology are being correlated in several different conditions: specifically in tropical sprue before and after treatment and in iron deficiency anemia.

(U) Approach - Gastrointestinal function is assessed by the use of a battery of tests including fat balance, D (+) xylose tolerance, Vitamin A tolerance, radioactive B_{12} and iron absorption studies, enzyme assays on intestinal biopsy material, and an indwelling double lumen tube technique utilizing polyethylene glycol as a non-absorbable marker. Gastrointestinal histology is evaluated by obtaining mucosal biopsies from various sites in the G.I. tract using the Crosby capsule.

The major technical problem is properly locating the position of the tubes used. The work would be greatly facilitated if a fluoroscope were located on our Ward.

(U) Progress - During this time the following results have been obtained:

25

1. In untreated tropical sprue a good correlation exists between histological appearance of the intestinal mucosa and tests of intestinal absorption.

2. A new classification has been described for the pathology of the jejunal mucosa. This work is helpful in clarifying the development of the jejunal lesion in tropical sprue and adds valuable information on the intestinal response to injury.

3. The proximal half of the intestine absorbs iron more efficiently than the distal half.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
"NA"	"NA"

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands)	36
CFY+1	

DD FORM 1 AUG 64 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A014501B71F: Research in Biomedical Sciences

Task No. 3A014501B71F 02: Internal Medicine

Work Unit No. B71F 02 37: Gastrointestinal Function and Histology (PR)

Reporting Installation: U. S. Army Tropical Research Medical Laboratory
APO New York 09851

Division: Department of Medicine

Period Covered by Report: 1 July 1964 - 30 June 1965

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Report Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

BODY OF REPORT

Project No. 3A014501B71F: Research in Biomedical Sciences

Task No. 3A014501B71F 02: Internal Medicine

Work Unit No. B71F 02 37: Gastrointestinal Function and Histology (PR)

Study 1. Effect of folic acid and vitamin B_{12} on the jejunal lesion of tropical sprue: A comparative morphologic study.

Progress:

Pre-treatment and multiple post-treatment jejunal biopsies from 7 folic acid treated patients and 8 vitamin B_{12} treated patients comprise the material used in this study. The parameters measured on a total of 60 biopsies included: overall mucosal thickness, total epithelial cell population counts, crypt cell nuclear size, argentaffin cell counts, and mitosis indexes. The data suggests that: a) the more severe lesions responded more dramatically than milder lesions to either form of therapy, b) neither folic acid or vitamin B_{12} are entirely effective in restoring the mucosa to a completely normal state, and c) there are slight differences in mucosal response between folic acid and vitamin B_{12} treatment, that of folic acid appearing to be more constant. Preparation of a manuscript and compilation of the data into chart form is now in progress.

Summary:

Patients whose jejunal biopsies have severe abnormalities responded with a more dramatic improvement of the histologic picture but neither folic acid or Vitamin B_{12} restored the mucosa to a completely normal state.

Study 2. Electron microscopic study of the jejunal mucosa in tropical sprue.

Progress:

The collection and processing of tissue from selected patients with tropical sprue is underway. The protocol outlining this project calls for 22 biopsies, of which 15 have already been acquisitioned. Patients have been carefully selected, i.e., where no previous treatment can be established with complete certainty, where no complicating factors such as intestinal parasitism or exogenous malnutrition are present, etc. Preliminary study of the ultrastructure of this tissue discloses definite morphologic abnormalities, the nature of which remain unclear at this early stage.

Summary:

Preliminary observations on 15 biopsies indicate that all exhibit definite abnormalities.

Study 3. Clinico-pathologic correlations in patients with tropical sprue.

Progress:

A study begun in 1963-64 on intestinal biopsy material from 150 patients with tropical sprue has continued. Earlier observations on the close correlation between pathologic abnormality and severity of clinical illness in untreated patients have been confirmed by subsequent examinations.

Summary:

The correlation between histologic findings and severity of clinical disease in tropical sprue has been established.

Study 4. Iron absorption in tropical sprue.

Progress:

Thirty five (35) studies of iron absorption have been done in patients with tropical sprue using a 5 mg dose of ferrous sulfate tagged with Fe^{59}. Mean absorption following this dose in 8 normal subjects was 19% with a range of 10 to 31%. Absorption in the patients with sprue varied from 0 to 80%. The most interesting finding was greater than normal iron absorption in many of the patients with steatorrhea associated with tropical sprue.

Summary:

Patients with tropical sprue may have decreased or increased absorption of iron. Interestingly, many of the patients with steatorrhea have increased absorption.

Study 5. Intestinal absorption of mono- and disaccharides in tropical sprue.

Progress:

Methods have been set up for the specific enzymatic measurement of various mono- and disaccharides and absorption studies by the use of a double-lumen tube infusion technique have been carried out in normal patients and in 10 patients with tropical sprue. It was believed that patients with intestinal mucosal damage might have more difficulty

handling disaccharides than monosaccharides since they must be hydrolyzed before being absorbed. However, reduction of monosaccharide absorption was found to be as severe as the lowering of disaccharide absorption. In addition, hydrolysis of lactose appeared to be decreased more (-67%) than that for sucrose (-47%) or maltose (-38%).

Summary:

Patients with malabsorption due to tropical sprue have a marked impairment in absorption of both mono- and disaccharides. There appeared to be particular difficulty in hydrolyzing and absorbing lactose.

Study 6. Intestinal disaccharidases in tropical sprue.

Progress:

The quantitative determination of sugar hydrolyzing enzymes (disaccharidases) has been carried out on the intestinal mucosa of patients with tropical sprue in various stages of severity and an attempt has been made to correlate enzyme deficiency with clinical symptoms, laboratory tests, and pathologic findings on biopsies. Previous studies in this laboratory have shown some correlation between the severity of the disease and reduction of activity of these enzymes. This work has been extended to 40 patients on long term treatment for tropical sprue. Surprisingly, there is a high incidence of lactase deficiency in patients who are clinically well and who have little if any pathologic changes in their intestinal mucosa. Further studies are in progress to work out the cause and effect relationships of this interesting phenomenon.

Summary:

Intestinal lactase deficiency was common in patients with tropical sprue although they were clinically well and their mucosa show little or no pathological change.

Study 7. Protein content vs. wet weight as basis for disaccharidase assays.

Progress:

At present two bases are commonly used for expressing disaccharidase activities: (1) units/gm protein, and (2) units/gm wet weight.

In many instances the biopsy is stored at -20°C for varying periods of time before analysis. The statement is often made that this storage does not lead to a change in enzyme activity. This has been found to be not the case. The biopsies were dissected immediately upon removal from patients. One piece was analysed immediately. The other half was frozen, stored in a closed tube at -20°C for the desired length of time, and then

analysed. Preliminary data indicate that using wet weight as the basis, the enzyme activities can increase 100% or more after only one month of storage at -20°C. Presumably this increase reflects a loss of water from the biopsy by sublimation of the frozen tissue water. With protein the change is variable and usually less than ±20% for the same storage period.

Summary:

The wet weight basis for expressing intestinal disaccharidases was found to be unreliable when specimens were frozen for 1 month.

Study 8. Carbohydrate absorption in children.

Progress:

A double-lumen polyvinyl tube was used for study of carbohydrate absorption in infants and children. Hydrolysis and absorption of the disaccharides lactose and sucrose were determined. Polyethylene glycol was the nonabsorbable reference substance. Specific analyses for the sugars were done enzymatically. In several children studied thus far, the tube has reached the level of the jejunum in 12 to 18 hrs. and infusions of approximately 2 liters have been given without untoward effect. The procedure may be useful in studying disaccharide absorption and may have further applications in other areas.

Summary:

Preliminary studies of small intestinal intubation in children suggest that it may be possible to evaluate disaccharide absorption in young children by an infusion technique.

Study 9. Colonic motility in acute infantile diarrhea.

Progress:

Studies have been started in collaboration with investigations from the Childrens Hospital of Philadelphia to determine the motility of the colon in infants with acute diarrhea during acute illness and after recovery. Children at the San Juan Municipal Hospital are being studied in the TRML Unit at this hospital using a Four-Channel Sanborn Recorder with two No. 10 French catheters inserted 6 and 12 cm. from the rectum. Recordings are taken in a tranquil environment while fasting and after milk feeding. Preliminary observations suggest that children 6 months to 4 years of age have a diminished motility pattern, whereas infants under 6 months may have an increased motility pattern.

Summary:

Preliminary studies have shown both increase and decrease in the motility pattern, depending upon the age of the child.

Study 10 Use of the Crosby-Kugler intestinal biopsy capsule in small and undernourished children.

Progress:

As an ancillary study to the analysis of infants with subacute and chronic diarrhea, the child-size Crosby-Kugler intestinal biopsy capsule has been evaluated in 50 children. Results show a perforation rate of 5% in about 100 biopsies. All perforations have been in severely emaciated children, usually less than 12 months of age or less than 9 kg. in weight. The standard child size capsule should therefore be used with care in such patients. Preliminary evaluation of a modified capsule having a 1.5 mm hole has shown that usable tissue can be obtained with such an instrument. It is believed this modification will render the capsule safer, but the limits of safety have not been defined and must await wider clinical use of the modified capsule.

Summary:

There was 5% incidence of perforation in emaciated children who are less than 1 year old or under 9 kg. in weight.

Study 11. Studies in dengue fever.

Progress:

Eighteen patients with suspected dengue fever were studied on the Metabolic Ward during 1963-64. Intestinal biopsies showed that 7 of these patients exhibited abnormalities of the jejunal mucosa; these patients were brought back for repeat biopsies. There was a definite improvement in the histological appearance of the mucosa. Serological proof of dengue virus infection is still pending.

Summary:

Attempts have been made to relate the histologic appearance of the jejunal biopsy to the clinical state of disease presumably associated with dengue.

Study 12. Serum immunoelectrophoresis in diarrhea in children.

Progress:

Serum from 85 children with diarrhea and intestinal symptoms of varying degree was tested against anti-human serum from goat by conventional slide immunoelectrophoretic techniques. There was no significant difference from the normal pattern in any instance.

Summary:

Eighty-five children with varying degrees of intestinal signs and symptoms showed no differences from a normal immunoelectrophoretic pattern.

Study 13. Precipitating antibodies to food antigens in the Puerto Rican diet.

Progress:

Antigens were made to various foods which are prevalent in the Puerto Rican diet. These antigens were those used in double-diffusion in agar tests in an attempt to detect precipitating antibodies in the sera of diarrhea patients.

Glass microscope slides are prepared with a thin layer of agar and a plastic matrix on top of the agar. An aliquot of antigen suspension is introduced into the agar layer through a central well in the matrix and sera to be tested are put into peripheral wells. These slides are then held at room temperature in a humid atmosphere for 48 hrs., at which time the matrix is removed and the slides are read for visible lines of precipitation occurring between the wells.

Eight-thousand eight hundred slide tests were performed on 601 sera. Approximately fourteen percent of the sera had precipitating antibodies to at least one food antigen.

Summary:

Fourteen percent (14%) of 601 sera from diarrhea cases had precipitating antibodies to one or more food antigens.

Study 14. Experimental malabsorption in animals.

Progress:

The attempt to produce an animal model for the study of malabsorption has continued. Mice are made deficient by feeding a diet deficient in folic acid. The mice are started on the deficient diet at the time of weaning; mice over 10 grams in body weight do not become deficient. Parameters studied are growth curve serum volate, whole blood folate, blood morphology, bone marrow morphology, fat absorption, xylose absorption, histopathology, formiminoglutamic acid excretion (FIGLU) and treatment with parenteral folic acid. Growth curves of the deficient mice are characterized by slow growth from the end of the second week, followed by weight loss after $3\frac{1}{2}$ to 4 weeks. Food consumption is somewhat reduced in the deficient groups.

Although serum folate is very low in the deficient mice (average 14

micromilligrams/cc), as compared to control mice (37 micromilligrams/cc) whole blood folate was found to show no difference. In the deficient mice values ranged from 80 to 260 micromilligrams/cc. Control values ranged from 95 to 320 micromilligrams/cc. Hence, no real difference exists. These values were obtained from blood frozen 3-6 months before the assays were made. Whole blood folate levels are being determined using fresh blood.

Blood counts show a slight decrease in granulocytes but bone marrow has not confirmed this change.

Summary:

Studies of folic acid deficiency in mice are being continued. No evidence of malabsorption has yet been found.

Study 15. Malabsorption in dogs.

Progress:

Contacts with the island veterinarians failed to produce dogs with histories of chronic diarrhea or steatorrhea without obvious causation such as heavy parasite loads or pancreatic and/or hepatic dyscrasias.

Summary:

An attempt has been made without success to find dogs with evidence of actual intestinal malabsorption.

Study 16. Infectious anemias of domestic animals.

Progress:

In an attempt to furnish a model for studying the effects of chronic anemia on such phenomena as iron absorption and uptake, folic acid utilization and associated intestinal lesions, various spontaneous protozoan diseases in dogs, cats, and one horse were studied. No gut morphological changes were found and other studies are waiting for more controlled conditions. Cases are infrequent, hard to maintain at a consistent and/or chronic level and present animal facilities are inadequate for experimental infections.

Summary:

Anemia of protozoan origin have been evaluated as possible models for the study of iron absorption, folic acid utilization and gut morphology. Results are, as yet, inconclusive.

Study 17. Chromosome pattern of the British West Indies monkey, Cercopithecus sabeus aethiops.

Progress:

Using peripheral blood leukocyte cultures for somatic chromosome analysis, samples were taken from both the B.W.I. monkeys and monkeys from West Africa. These animals were both similar in pattern and number of the somatic chromosomes, 2n = 60 with three pairs of acrocentric chromosomes.

Summary:

The chromosome pattern and number of the green guenon monkey from the Islands of St. Kitts and Nevis British West Indies is the same as the African Green Monkey from West Africa (2 n = 60) with 3 pairs of acrocentric chromosomes.

Study 18. Influence of high level antibiotic and/or sulfa intake on gut cell turn-over time.

Progress:

Four groups of mice, 15 mice/group, received for 14 days various antibiotics or sulfas administered in their drinking water.

Group I - Controls
Group II - Aureomycin, 125 mg/lb/day
Group III - Sulfasuxidine, 4.8 gm/lb/day
Group IV - Penicillin V, 150,000 units/lb/day

Mice from each group were given tritiated thymidine i.p. (7 microcuries/gram) then killed at intervals of 4 hours through 72 hours, the tissues were routinely processed for sectioning; AR-10 autoradiographic stripping film or Kodak liquid emulsion NTB-2 was applied and the slides exposed for periods of about 8-10 weeks. Gut cell turn-over time is being measured by determining the length of time required for maturation of a crypt cell through its migration to the villus tip. Results are pending.

Summary:

Mice were placed on high levels of either antibiotics or a soluble sulfa. The gut cell turn-over time was measured by labeling with tritiated thymidine, the gut cell epithelium while undergoing division in the crypts then the labelled cell is traced and timed as it migrates towards the villus tip and cellular maturation.

Publications:

1. Pathology of the jejunal mucosa in tropical sprue. VIIIth Conference on Intestinal Malabsorption and Allied Hematologic Problems, San Juan, P. R., March 1964. V. Swanson and R. Thomassen. Am. J. Dig. Dis. 9, 772-774. 1964 (Abstract).

2. The importance and reliability of the roentgenographic examination of the small bowel in patients with tropical sprue. W. Caldwell, V. Swanson, and T. Bayless. Radiology 84, 227-240. 1965.

3. Pathology of the jejunal mucosa in tropical sprue. V. Swanson, and R. Thomassen. Am. J. Path. 46, 511-552. 1965.

4. Newer developments in tropical sprue. V. Swanson. Bull. Internat. Acad. Path. 6, 35-37. 1965.

5. Mycologic study of jejunal biopsies from tropical sprue patients. V. Swanson, L. Haley, and M. S. Wheby. Am. J. Trop. Med. Hyg. In Press.

6. Equine piroplasmosis in Puerto Rico. Thomas C. McChesney, and L. P. Jones. J.A.V.M.A. 146, 23. 1965.

7. Canine piroplasmosis: A spontaneous outbreak. L. P. Jones, and J. M. Morris (In Preparation).

8. Chromosome number and pattern of the British West Indies Green Guenon. L. P. Jones. (In Preparation).

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 6406	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
1 07 65	A. New	U U (RPT WRK)	"NA"	NL	A. Work Unit

10a. CURRENT NUMBER/CODE	10b. PRIOR NUMBER/CODE
61145011 3A014501B71R 02 072	61145011 3A014501B71F 02 38

11. TITLE:

(U) Metabolic Changes in Malnutrition (PR)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
003500 Clinical Medicine	10 63	"NA"	Other | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C	"NA"	PRIOR FY 65	2	125
		CURRENT FY 66	2	231

19. GOV'T LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
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21. TECHNOLOGY UTILIZATION	22. COORDINATION
"NA"	"NA"

23. KEYWORDS: Metabolic, Enteron, Nutrition, Morphology, Biopsy, Enzyme, Biopsy Capsule, Mucosa

24. (U) Tech Objective - The metabolic function of the enteron and nutritional status are intimately related to growth and ultimate functional level; the relation of enteric function, nutritional status and enteric morphology is under investigation in children and adults.

25. (U) Approach - The approach has been to define problems and develop suitable methods. Suitability of the intestinal biopsy capsule for small emaciated infants is being investigated; feasibility of use of smaller tissue samples for enzyme analysis is being investigated. Disappearance rates of dietary sugars are being studied. Histopathological correlates of the intestinal biopsy is being studied.

26. (U) Progress - A safer modification of the intestinal biopsy capsule has been tested and found satisfactory. It has been found feasible to do enzyme analysis on 3 mgm samples of mucosa. The histological appearance of the jejunal mucosa of a group of children of marginal nutritional status has been described. Low lactase levels are frequently found among individuals of marginal nutritional status.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
"NA"	"NA"

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A014501B71F: Research in Biomedical Sciences

Task No. 3A014501B71F 02: Internal Medicine

Work Unit No. B71F 02 38: Metabolic Changes in Malnutrition (PR)

Reporting Installation: U. S. Army Tropical Research Medical Laboratory
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Division: Department of Medicine

Period Covered by Report: 1 July 1964 - 30 June 1965

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Report Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

BODY OF REPORT

Project No. 3A014501B71F: Research in Biomedical Sciences

Task No. 3A014501B71F 02: Internal Medicine

Work Unit No. B71F 02 38: Metabolic Changes in Malnutrition (PR)

Study 1. Chronic diarrhea and malabsorption in Puerto Rican children.

Progress:

Fifty Puerto Rican children mainly from poor families (age 4 months to 12 years, median 36 months) have been studied to establish a clinical and pathological classification of chronic diarrhea. All had at least one proximal jejunal biopsy. All had at least one, usually more, six-day fecal fat determinations. At least 3 stools were examined quantitatively for evidence of parasites. An hematologic diagnosis was established by examination of peripheral smears, bone marrows and serum iron and iron binding capacity. Twenty-five children have been followed for over one year.

Virtually all had some degree of iron deficiency and 8 had initial hemoglobins less than 9 gms. Jejunal biopsies were classified as normal (4), mild nonspecific jejunitis (16), and definite changes suggestive of malabsorption (30). There was no clear correlation between the degree of iron deficiency and the intestinal biopsy. Twenty-five of the 50 children excreted greater than 5.5 gms. fecal fat; 8 excreted 4.5 - 5.5 gms. and 17 excreted less than 4.5 gms.

Jejunal mucosa was assayed for lactase, invertase, isomaltase and maltase in 26 of the children. The median lactase content was 1.6 M/min/gm wet weight (range 0 - 9.3) and the mean ratio of invertase to lactase was 5.7 (range 1.1 - 16.8, median: 4.4).

Thus, in the San Juan area of Puerto Rico recurrent and chronic diarrhea is more common than in the United States. Ordinary bacterial pathogens are not commonly isolated from such patients; the most common parasitic infestation is trichuris. The majority of cases have mild to minimal steatorrhea associated with distinctly abnormal bowel movements and the etiology is unexplained.

Biopsies of the jejunum from these patients show a smooth continum of pathological change characterized by evidence of increased epithelial cell turnover and subacute or chronic inflammation. When these changes are moderate to severe in degree, steatorrhea is present.

About 40 biopsies from such patients have been analyzed for lactase, sucrase and maltase activity. Many have greatly reduced enzyme levels,

particularly lactase. The significance of this finding is under investigation and two specific points must be approached: 1) The incidence of reduced lactase levels of well nourished Puerto Rican children from hygienic homes who have not had recent intestinal symptoms and 2) Hydrolysis rates and jejunal disappearance rates from both normal and chronic diarrhea subjects.

There is no question that the more severe cases of steatorrhea have a histopathological picture resembling the lesion of tropical sprue in adults; whether one is justified in labeling the clinical syndrome tropical sprue remains debatable.

Summary:

Low grade steatorrhea and considerable jejunal inflammation are common among children with chronic diarrhea.

Study 2. Effect of the anticonvulsant drugs diphenylhydantoin and phenobarbital on tetany and the Trousseau phenomenon.

Progress:

The high incidence of convulsive seizures in hypoparathyroidism and hypocalcemic states make it essential to rule out the presence of these disorders in evaluating seizure patients. This is done by applying tests for latent tetany, the Trousseau, Trousseau-von Bonsdorff and Chvostek tests, and obtaining chemical determinations of serum calcium and phosphorus. The fact that serum calcium determinations are often notoriously unreliable places added importance on the clinical tests for latent tetany. Because anticonvulsant drugs are often prescribed after an initial seizure the authors have evaluated their effect upon the clinical signs for latent tetany and serum mineral values. Nine patients have been studied, 5 untreated hypoparathyroid patients, 2 hypoparathyroid subjects with a persistent positive Trousseau on Vitamin D and 2 subjects with idiopathic latent tetany. Oral diphenylhydantoin with or without phenobarbital eliminated the positive Trousseau in all hypoparathyroid patients and also demonstrated a potent acute effect by i.v. administration. Oral phenobarbital was less effective, eliminating these signs in only one subject. The 2 patients with idiopathic latent tetany proved more resistant to both drugs by any route. Chvostek's sign was less effected and serum calcium, magnesium and phosphorus never changed significantly. These drugs may eliminate an important clue to the diagnosis of hypoparathyroidism in seizure patients. Their mode of action is under further consideration.

Summary:

Diphenylhydantoin and to a much lesser extent phenobarbital, depress the Trousseau, Trousseau-von Bonsdorff and Chvostek's responses in hypocalcemic tetany.

Study 3. Dystonic neuromuscular reactions to prochlorperazine.

Progress:

The increased excitability of the nervous system in hypocalcemia is well known, with convulsive seizures and tetany representing cortical and peripheral manifestations. The basal ganglia are also involved in chronic hypocalcemia with calcific precipitates and occasional motility disorders resulting. The occurrence of a severe neuromuscular dystonic reaction involving the head and neck in a post-operative hypoparathyroid patient followed a small i.m. dose of prochlorperazine prompted a test of this drug in other hypoparathyroid subjects. Five hypoparathyroid subjects all developed reactions, and in 2 of 3 subjects the reactions again occurred with larger dosages of prochlorperazine when the subjects were normocalcemic on Vitamin D. This is startling when compared with the usual 2-3% incidence of these reactions in subjects receiving phenothiazine derivatives. There are no previous reports of such reactions in hypoparathyroid patients in the literature, and indeed, no other underlying disease so predisposes to this reaction. Such a drug reaction may prove an interesting clue to underlying hypoparathyroidism, and it may provide a reliable way of producing the reaction for further neurophysiologic study. Such considerations are under further study.

Summary:

All of five hypocalcemic hypoparathyroid subjects developed dystonic reactions when initially challenged with small doses of prochlorperazine.

Study 4. Does hypocalcemia cause malabsorption?

Progress:

The finding of steatorrhea in cases of idiopathic hypoparathyroidism has been noted too often in the literature to be a chance association raising the possibility that prolonged hypocalcemia may provoke intestinal malabsorption. In an attempt to answer this question studies have continued in hypoparathyroid patients before and after treatment of Vitamin D_2 utilizing jejunal biopsy, small bowel series, and the usual laboratory tests for malabsorption. A total of 13 patients have been studied completely, including 5 idiopathic and 6 post-operative hypoparathyroid subjects, 1 pseudohypoparathyroid patient and 1 patient hypocalcemic on two occasions following removal of a parathyroid carcinoma. Three subjects have again been studied while off Vitamin D after previous evaluation before and after treatment. None of these untreated patients have shown dramatic malabsorption although a significant number have shown a low grade steatorrhea, impaired xylose excretion, poor Vitamin A absorption or low carotene, and small bowel series have shown mild to moderate changes in a number of patients; jejunal biopsies have shown nonspecific changes. All these abnormalities tended to revert toward normal with normocalcemia on Vitamin D. One post-operative patient currently off

Vitamin D and severely hypocalcemic has shown steatorrhea averaging 10 gms. daily, and abnormal small bowel pattern but normal 25 gm. xylose excretion; this is the only subject to show such disparity, and she is to return for further studies. Because the subjects were asymptomatic from the standpoint of malabsorption, a control series of asymptomatic Puerto Rican subjects are being similarly evaluated.

Summary:

A significant number of patients with hypocalcemia showed a mild degree of malabsorption. Study of a normocalcemic Puerto Rican control group is underway.

Study 5. Factitious hypoglycemia in thrombocythemia.

Progress:

The finding of repeated extremely low blood sugars in a symptomatic postpartum patient ultimately shown to have thrombocythemia prompted a study of glucose consumption by platelets. Blood from this patient and another subject with a comparable elevation in platelets due to polycythemia vera were studied with and without the addition of sodium fluoride and blood glucose determinations made over a 5-hour period. Compared to normal controls done simultaneously, blood from these two subjects showed a more rapid consumption of glucose when incubated at 37°C or room temperature. Fluoridinated tubes as prepared in the main hospital laboratory did not completely block this glucose consumption but when a saturated solution of sodium fluoride was used in liquid form at 5 mg/ml of blood the glucose consumption was blocked. This factitious hypoglycemia was apparently caused by excessive glucose consumption by platelets incompletely blocked by fluoride, and corresponds to the well known factitious hypoglycemia of leukemia.

Summary:

Two patients with thromocythemia showed a rapid consumption of glucose which was not completely blocked unless a saturated solution of sodium fluoride was used as a preservative.

Study 6. Estimation of calcium absorption utilizing sustained infusion of isotopic calcium through a double-lumen tube.

Progress:

Ca_{47} or Ca_{45} with varying concentrations of added stable calcium carrier in normal saline has been infused into the upper duodenum through a double-lumen tube and collections taken 50 cm distally. PEG has been used as a non-absorbable marker. Three subjects have been studied to date, a normal, a patient with untreated pseudohypoparathyroidism and a patient with Vitamin D

resistant rickets whose primary defect is considered to be isolated calcium malabsorption. It was thought that secondary hyperparathyroidism might be increasing calcium absorption in the latter subject, since primary hyperparathyroidism is associated with increased calcium absorption; to eliminate the influence of secondary hyperparathyroidism on calcium absorption, the patient received calcium infusions 25 mg/kg over a 12-hour period on three successive days with repeat of the isotopic calcium tube study on the succeeding day. The pseudohypoparathyroid patient was similarly studied. Results are under evaluation, and further studies are planned using this method of measuring calcium absorption in normal subjects and those with disorders effecting calcium metabolism or absorption.

Summary:

Studies have been initiated on the estimation of calcium absorption from the human intestine by the use of an infusion technique. Preliminary results are being evaluated before the study is extended to other subjects.

Study 7. Evaluation of calcium absorption by oral administration of Ca^{47} in normal subjects and patients with tropical sprue.

Progress:

Persistent calcium malabsorption results in hypocalcemia and severe bone disease, but these are late, uncommon manifestations, and an early and more sensitive index of malabsorption is needed. Calcium balance studies do not satisfy this need, since they are complex and subject to many sources of error.

Therefore, orally administered Ca^{47} with 50 mg calcium carrier as $CaCl_2$ has been evaluated as an index of absorption; plasma activity was measured at 2 hours, urine for 4 days and feces until negligible Ca^{47} activity remained. Stable calcium, phosphorus and creatinine was measured in plasma and urine, and a complete battery of absorption tests including jejunal biopsy were used to correlate calcium absorption with other parameters of absorption.

Twenty normal subjects have been compared to 17 patients with untreated or ineffectively treated tropical sprue, 7 patients recovering from tropical sprue on Vitamin B_{12} or folic acid treatment, and 9 patients undergoing recovery on a gluten-free diet, antibiotics spontaneously.

Although the usual parameters used to measure calcium malabsorption, (serum calcium, phosphorus and alkaline phosphatase, 24-hour urinary calcium excretion, x-ray evidence of osteomalacia) did not separate normal subjects from those with tropical sprue, Ca^{47} absorption clearly distinguished these groups.

Calcium hyper-absorption was also demonstrated in primary hyperparathyroidism and hypoparathyroid patients treated with Vitamin D_2. In the

latter 5 subjects Ca^{47} measurements before and after Vitamin D_2 showed dramatic change.

Studies have also been done in patients with hyper or hypothyroidism, Vitamin D-resistant rickets, osteoporosis, probable Cushings' syndrome and acromegaly.

Serial forearm counts after oral Ca^{47} are being compared with absorption studies since this is a bone-seeking isotope.

Summary:

Calcium^{47} absorption has been shown to be a sensitive indicator of early calcium malabsorption.

Study 8. Urinary amino acids in patients with tropical sprue.

Progress:

An iron exchange type of automatic amino acid analyzer was used to determine urinary amino acids in several treated and untreated tropical sprue patients. In the cases studied, no significant pattern of amino acids was noted, nor were excessive quantities of any amino acid observed. This could be due partly to the wide variations in normal urinary amino acid excretion.

Summary:

An amino acid analyzer was used to determine the amino acids excreted by patients with treated and untreated tropical sprue. Excretion appeared to be normal.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6400	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
1 07 65	A. New	U / U	"NA"	NL	A. Work Unit

10a. CURRENT NUMBER/CODE: 61145011 3A014501B71P 10 030

10b. PRIOR NUMBER CODE: 61145011 3A014501B71P 11 01

11. TITLE: (U) Biology of Schistosomiasis and Other Parasitoses (PR)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 Biology	01 63	"NA"	Other | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C	"NA"	PRIOR FY 65	2	25
		CURRENT FY 66	1	14

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
"NA"	"NA"

23. KEYWORDS: Schistosomiasis, Cercopithecus, rhesus, Co^{60} irradiated cercariae

24. (U) Tech Objective - The purpose of a broad study of schistosomiasis in a variety of experimental hosts is to reveal the animal whose response to the infection is the same as in the human. Work here is now centered on the Cercopithecus monkey which in several important respects serves this purpose better than the rhesus monkey.

25. (U) Approach - Animals are given single, graded exposures or multiple small exposures. One group is being given multiple exposures with Co^{60} irradiated cercariae. Appropriate tests in use to collect data on response of animals to infection over an extended period of time. Ultimately a determination will be made of response to reinfection. Generally, parallel studies also in progress in mice.

(U) Progress - Monkey exposures have been or are being accomplished. Observations are being made as scheduled. These include serum electrophoresis, serological tests, and various liver function tests.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
"NA"	"NA"

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A014501B71P: Basic Research in Support of Military Medicine

Task No. 3A014501B71P 11: Zoological Sciences

Work Unit No. B71P 11 01: Biology of Schistosomiasis and Other Parasitoses (IX)

Reporting Installation: U. S. Army Tropical Research Medical Laboratory
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Period Covered by Report: 1 July 1964 - 30 June 1965

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Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

BODY OF REPORT

Project No. 3A014501B71P: Basic Research in Support of Military Medicine

Task No. 3A014501B71P 11: Zoological Sciences

Work Unit No. B71P 11 01: Biology of Schistosomiasis and Other Parasitoses (PR)

Study 1. Reduction in urinary protein excretion in mice infected with Schistosoma mansoni.

Progress:

Work previously reported indicated that infection with S. mansoni presumably caused a marked reduction in excretion of urinary proteins in mice in about the 4th week of infection. This observation had been based on randomly collected urines, but since it had a possible diagnostic application it was desirable to continue the study on an systematic basis.

Observations have now been made on weekly 24-hour urine collections from mice prior to exposure and in the 3rd to 7th week following exposure to 100 bisexual cercariae. Data from uninfected control mice were obtained for comparison. Electrophoresis of urine was done with 20 lambda volumes; and quantitative protein determinations were made on 1 ml portions of 24-hour specimens with a technique that had been devised here.

The mean urine outputs for the 24 hours prior to exposure were 2.54 ml in the infected group and 2.33 ml for controls. Subsequently, mean 24-hour volumes for infected mice ranged between 2.22 ml and 2.92 ml in the 3rd through 6th week of infection, and then decreased abruptly to 1.41 ml in the 7th week. The corresponding protein output was 3.46 to 6.27 mg/ml. The protein concentrations per 24-hour collection were 9.27 mg to 13.66 mg in weeks 3 to 6 but only 2.01 mg in week 7. Control mice maintained generally regular outputs of 2.51 ml urine/24 hours, 5.26 mg protein/ml, and 13.06 mg/24 hours.

Summary:

Although certain other aspects of this study must still be worked out, e.g., the effect of worm burden on reduction of urinary protein, it appears that schistosomiasis produces changes in the mouse host that may be reflected in urinary disturbances.

Study 2. Effect of Fuadin on excretion of urinary protein in mice.

Progress:

Mice infected with S. mansoni were treated with Fuadin according to

a standard 3-week regimen in order to determine if excretion of urinary protein in infected animals would return to normal levels after cure. Electrophoresis and quantitative protein determinations were performed weekly on 24-hour urine samples prior to, during and after treatment. Uninfected Fuadin-treated mice showed protein bands throughout the experiment. Urinary protein decreased during treatment and then increased to a peak in the 3rd post-treatment week, after which it started to decrease again. In the infected-treated group, 6 of 14 mice showed light bands in the first week of treatment and only 1 of 12 showed bands in the 2nd week, but in the 3rd week of treatment 3 of 11 had bands. By the 2nd post-treatment week all mice showed bands. Bands in this group were fainter than those in the uninfected-treated group at all times. Excretion of urinary protein in the infected-treated group began to increase in the 2nd week of treatment but at no time did the concentration equal that in the uninfected group. A parallel group of infected mice (untreated) were examined in the 4th post-treatment week; only 5 of 15 mice showed bands and mean protein excretion was less than half that in the treated group.

Summary:

Following treatment with a standard course of Fuadin, excretion of urinary protein in mice infected with S. mansoni increased significantly and protein bands again became demonstrable in almost all animals before treatment was completed.

Study 3. A new technique for quantitative measurement of urinary protein.

Progress:

Studies of the effect of schistosomiasis mansoni on the excretion of urinary protein by mice have necessitated development of an accurate method for measurement of the protein in small samples of urine. The currently available Biuret procedure was not feasible because there seemed to be interferring substance(s) in the urine of infected mice that affected color development. The new technique can be used with urine samples 0.25 ml or less in volume and has been shown to yield reliable and reproducible results.

Summary:

A technique has been devised to replace the Biuret procedure that is not usable because of interferring substances(s) in urine from infected mice.

Study 4. Acquired resistance to S. mansoni elicited in mice by intraperitoneal and percutaneous routes of infection. I. Effect of route of challenge infection on demonstration of resistance.

Progress:

An earlier study indicated that a higher level of acquired resistance was elicited by multiple light exposures with the IP route of infection, as compared with the more conventional PC route, and further, that IP challenge following IP immunization demonstrated a higher level of resistance than PC challenge. The present experiment was performed to confirm this observation and to compare levels of resistance after 2 periods of immunizing infection with a single exposure. The experiment was done in 2 parts. In the first, 100 cercariae were used for the immunizing infection, but because of excessive losses of mice in some groups this number was reduced to 50 for the second part. For each replicate, however, challenge was made with 100 cercariae either 4 weeks or 8 weeks after the initial exposure, and by both the IP and the PC routes. Therefore, the outline of the experiment was: 2 routes of immune X 2 routes of chall. X 2 periods of immunization X 2 parts of the experiment.

Levels of resistance elicited by 50 cercariae were strikingly similar to those given by 100 cercariae. Thus, in 6 instances in which a significant level was elicited by 100 cercariae (50% reduction or more of challenge infection worm burden) a level nearly as high was also induced by 50 cercariae in all cases but one. In 2 other cases either exposure route resulted in a negligible degree of resistance. Following IP immunization, 87-100% reduction in challenge infection worm burdens occurred when challenge was administered by the IP route either 4 weeks or 8 weeks after immunization. If, however, the PC route was used for challenge, worm burden reduction was negligible after 4 weeks immunization and not especially high after 8 weeks. Results with PC immunization were somewhat different. When challenge was given by the PC route, worm burden reduction was greater after 8 weeks immunization than after 4 weeks, but not significantly so. But with IP challenge there was negligible reduction after 4 weeks and 90-100% reduction after 8 weeks.

Summary:

Results of a factorial design experiment show that while the IP and PC-routes of infection elicit comparable levels of resistance, this resistance is demonstrable earlier when the homologous route is used for challenge.

Study 5. Acquired resistance to S. mansoni elicited in mice by intraperitoneal and percutaneous routes of infection. II. A comparison of levels of resistance elicited by both routes 4, 8, and 12 weeks after primary infection.

Progress:

The original objective of this experiment was to compare the effect of immunizing infections with 50 cercariae administered by either the IP or the PC route on worm burdens resulting from challenge with 100 cercariae administered 4, 8, or 12 weeks thereafter by the homologous route. Results indicated that either route of immunization elicited a reasonably high level of resistance after 4 weeks, but after 8 weeks the level with the IP route was higher, while after 12 weeks the PC route was better. When the experiment was repeated the IP route for immunization was shown to induce resistance that was as high after 4 weeks as after 8 and 12 weeks. The PC route elicited a negligible level after 4 weeks, a somewhat higher level after 8 weeks, and a level equal to that of the IP route after 12 weeks.

Tests between replicates indicated that reproducibility of results was not particularly well marked. In 6 combinations involving reduction of challenge infection worm burdens between replicates, there was agreement between replicates in only 3. In the 3 instances of disagreement the difference between values for the 2 replicates was as much as 4-fold in one case and 1½ in the other two.

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An experiment of this type involves at least 3 groups of animals: an immunized-challenged (ICh) group; immunizing controls (I); and challenge controls (ChC). These, with 2 routes of infection and 3 periods of immunization, make a total of 6 groups of each type to be compared between the two replicates. Considering first the ICh groups, in 2 out of 6 instances there was a significant difference ($P \leq .025$) between replicates. In neither case, however, was there a significant difference between I or ChC groups associated with these ICh groups. Yet, in one case worm burden reductions in the two replicates were 61.5% vs 84.6%, while in the other they were 73.6% vs 88%. In the other 4 instances in which significant differences did not occur between ICh groups in the replicates, worm burden reductions were: 61.9% vs 17.5%; 57.4% vs 40%; 51.4% vs 81.8%; and 78.2% vs 84.9%.

The I groups differed significantly between replicates in 2 instances also, and in neither were there significant differences within ICh and ChC groups. In one case worm burden reductions were 61.9% vs 17.5%, but in the other they were 78.2% and 84.9%.

ChC groups did not differ significantly between replicates in any instance.

Summary:

Poor reproducibility of results between 2 replicates of an experiment does not permit a conclusion relative to the rate of development of resistance following a single exposure to 50 cercariae via the IP or PC route. This observation again points out the need for replicated tests in studies based on conventional infection - challenge design.

Study 6. Studies on schistosomiasis in Cercopithecus monkeys: Evaluation of techniques used in recovery and quantitation of eggs.

Progress:

The techniques in use for recovering and quantitating Schistosoma mansoni eggs in feces are different than previously employed in related studies. Three procedures have been evaluated, including, 1) the Formalin-Ether dilution egg-counting technique (FE-D technique), for which the volume of feces is determined by centrifuging a comminuted, strained specimen for a standard time and speed, 2) the Formalin-Ether concentration procedure, using a buffered alcoholic solution (FE-BA technique), and 3) the examination of pooled fecal samples, collected weekly for bimonthly intervals, as a means of determining the pattern of egg-output.

Although comparisons were made with certain other procedures, the objective of this report is to evaluate the efficiency of the above techniques as they are performed at TRML.

Formalin-Ether dilution egg-counting procedure (FE-D technique):

1. Comminuted formalinized feces are strained through two (2) thicknesses of gauze into a graduated 15 ml centrifuged tube (after centrifugation there should be 0.5 to 1.0 ml of packed solids).

2. The feces are centrifuged for 5 minutes at 1500 rpm.

3. The volume of solids is accurately recorded and the supernate is discarded without loss of any sediment.

4. Exactly 14 parts of either, 10% formalin, or buffered alcoholic solution are added; thus the dilution is 1:15.

5. After thorough mixing, 0.15 ml is transferred by Stoll's pipette to a slide and covered with a 22 x 40 mm coverslip.

6. All the eggs are counted, and since the total volume was 15 and the aliquant 0.15, the dilution factor is 100. The computed figure represents eggs per ml of a standardized sample of strained, centrifuged, fecal sediment.

With this procedure, two technicians obtained consistent results, using two "tube samples" from the same stool and counting eggs in 10 aliquants, obtained by using Stoll's pipette. On a second stool, one technician, counting 10 aliquants from each of two "tube samples" again obtained consistent results. On a third stool, two technicians, each counting eggs in five aliquants from four "tube samples" obtained consistent results with both 0.15 and 0.075 ml of emulsion.

The efficiency of the Formalin-Ether concentration procedure using a buffered alcoholic solution (FE-BA technique).

This technique was described in the 1964 Annual Report. Egg counts were determined by means of the FE-D technique on 8 different stools from each of three Cercopithecus monkeys. Subsequently, each "tube sample" was concentrated by means of the FE-BA technique. The sediment recovered by the latter was diluted to 1.5 ml and an aliquant sample of 0.15 ml was taken by means of Stoll's pipette and egg counts were made (dilution factor = 10). Numbers obtained were similar to those provided by the FE-Dilution technique, indicating a good efficiency for the FE-BA procedure.

The examination of pooled samples of stools rather than each stool singly.

During periods of one month and 2 months, eight or nine stools were collected from each of three monkeys. Egg counts were made on each stool by the FE-D technique, and from each stool a sample of 2 ml was comminuted in 15 ml of formalin and added to a fecal pool for the respective monkey. When the pools were complete, a single "tube sample" of each was prepared and egg counts were made by the FE-D technique (2 or 3 aliquants per tube). The mean egg counts for the series of single stools for the three monkeys were 17.3, 13.8 and 13, while for their respective fecal pools, the counts were 16.3, 15.3 and 17.3. It appears that considerable time can be saved by using a pool of fecal samples collected over a 2-month period.

Summary:

Consistent results were obtained by paired technicians using the Formalin-Ether dilution egg counting technique. In comparison with this procedure the Formalin-Ether concentration technique, using a buffered alcoholic solution, showed high efficiency in recovering S. mansoni eggs. Instead of examining single stools, it proved feasible to pool 2 ml formalinized samples of a series of stools (1 per week for 2-month intervals) and then examine a single sample of the fecal pool.

Study 7. Studies on schistosomiasis in Cercopithecus monkeys: Pathobiological studies throughout the entire course of light infections.

Progress:

Whereas previous histopathological studies at TRML on schistosomiasis

mansoni in Cercopithecus have been limited either to lethal infections with which monkeys survived for about 2 months or heavy infections of several years duration, the current studies involve period observations throughout the entire course of light infections. Supportive biological observations will include routine fecal egg counts, terminal cumulations of eggs in the organs, and worm survival.

One Macaca and two Cercopithecus monkeys exposed to 100 cercariae will be necropsied after infection intervals of $1\frac{1}{2}$, 3, 6, 12, 24, 36, 48 and 60 months. At the time of necropsy manual worm recoveries will be made both frozen and fixed tissues will be collected, and organ egg counts will be made on tissue samples at various levels of the small and large intestines, liver, and all other organs that might contain worms and/or eggs. This data will be correlated with histopathological observations.

Summary:

A series of Cercopithecus monkeys are being exposed to 100 cercariae for histopathological studies throughout the entire course of the infection. Necropsies will be made $\frac{1}{2}$, 3, 6, 12, 24, 36, 48, and 60 months after exposure.

Study 8. Studies on schistosomiasis in Cercopithecus monkeys: Biological observations on egg production.

Progress:

Two ether-sedimentation techniques were used to determine the day of infection when eggs could first be recovered from the feces. The monkeys had received either 500, 200, 50 or 10 cercariae. Stool examinations were started on the 33rd day of infection using the AMS-III and the Formal-in-Ether procedures, the latter with a buffered alcoholic solution (FE-BA technique). These two concentration procedures were about equally effective. First egg recoveries were made on the same day by both techniques in the case of 11 monkeys, and the FE-BA and AMS-III each recovered eggs first on 13 and 7 monkeys, respectively.

For 13 Cercopithecus exposed to 500 cercariae the day of "first eggs" ranged from 36 to 40, with a mean of 38 days. Animals receiving only 200 cercariae gave a range of 39 to 41 days with a mean of 41.1 days. For 3 animals exposed to 50 cercariae the range was from 35 to 42 days, with a mean of 38. Three monkeys receiving only 10 cercariae were positive on the 39th, 42nd and 43rd days, for a mean of 41.3 days. The overall average for all Cercopithecus monkeys was 39.0 days. Of three Macaca mulatta monkeys, two were positive for the first time on the 39th and one on the 40th day. These animals had been exposed to 200 cercariae, so their average of 39.3 must be compared with 40.1 days for Cercopithecus monkeys exposed to 300 cercariae. It appears then that there is very little difference in the incubation period of Schistosoma mansoni in Macaca mulatta and Cercopithecus monkeys.

Mean egg-counts for each of 9 Cercopithecus monkeys exposed to 200 cercariae were as follows for weeks 7 through 15:

739	1089	406
1133	272	567
500	1050	644

Corresponding figures for three Macaca mulatta monkeys also exposed to 200 cercariae were;

133	250	294

and for 3 Cercopithecus monkeys receiving 50 cercariae each, the corresponding mean figures were:

100	128	206

From these figures it is apparent that egg counts for individual monkeys varied considerably, even though the animals were all exposed at the same time to the same collection of cercariae.

Mean egg counts for 9 monkeys collectively at weekly intervals from 7 through 17 were as follows:

Week	Mean Eggs	:	Week	Mean Eggs	:	Week	Mean Eggs
7	617	:	11	406	:	15	611
8	672	:	12	761	:	16	383
9	844	:	13	1133	:	17	383
10	722	:	14	633	:		

From these data it is apparent that near-maximum egg out-put prevailed within one week after eggs first appeared. It is believed that variations in egg counts through the first nine weeks were fortuitous. Further observations are necessary before significance can be attached to the decline noted in the 16th and 17th weeks.

Summary:

Two ether sedimentation techniques (FE-BA and AMS-III) were about equally effective in recovering "first" eggs. The mean day when stools became positive was the 39th day from exposure. The same was true for Macaca mulatta monkeys. For 9 monkeys exposed to 200 cercariae, there was considerable difference in egg-output (weeks 7-15). The egg counts for macaque monkeys were lower than the Cercopithecus. Even within one week from patency, egg-output was at a near-maximum level.

Study 9. Studies on schistosomiasis in Cercopithecus monkeys: Observations on acquired resistance resulting from antecedent infections.

Progress:

Observations continue on three (3) Cercopithecus monkeys, two of which have been exposed two times. The latter were exposed first in 1961 with 100 cercariae. After $2\frac{1}{2}$ years their egg counts had reached a low level. They were then reexposed to 250 cercariae and egg counts increased. A challenge control monkey without previous exposure discharged about the same number of eggs. Output of eggs since the second exposure has continued equally for all three animals for 16 months. Thus it appears that the Cercopithecus monkey is much less responsive against existing mature infections than Macaca mulatta monkeys and after an infection of $2\frac{1}{2}$ years there was little evidence of acquired resistance, which is also in contrast to the macaque monkey. The idea is entertained that these two species of monkeys should prove to be complementary experimental hosts for investigating the mechanisms of immunity against schistosomiasis, because of their contrasting reactions against the parasite.

Summary:

Two Cercopithecus monkeys showed renewed output to S. mansoni eggs after challenge made $2\frac{1}{2}$ years after the initial immunizing exposure. Egg output after challenge has persisted with only minimal reduction for 16 months.

Study 10. Studies on schistosomiasis in Cercopithecus monkeys: Normal serum protein patterns in comparison with Macaca mulatta monkeys.

Progress:

Preliminary to a study on changes in serum protein patterns resulting from infection with schistosomiasis mansoni, paper electrophoresis was carried out on normal sera from a number of rhesus monkeys and the St. Kitts Island and West African varieties of Cercopithecus sp. or green monkeys. The green monkeys invariably showed only one alpha fraction, in contrast with the two alphas (1 and 2) normally seen in rhesus serum. In addition, a difference in the beta fraction was seen, not only between the rhesus and green monkeys, but between the two varieties of green. Whereas the rhesus and West African green show 3 beta fractions (1, 2, and 3), the St. Kitts green shows only a two-band split, beta-1 being the absent component.

Summary:

A study of normal serum protein patterns in rhesus and 2 varieties of green monkeys shows that while the rhesus has 2 alpha fractions, the greens have only 1, and while the rhesus and the West African greens have 3 betas, the St. Kitts greens have only 2.

Study 11. Studies on schistosomiasis in Cercopithecus monkeys: Effects of infection on serum protein patterns in comparison with Macaca mulatta monkeys.

Progress:

Paper electrophoresis (veronal buffer, pH 8.6, ionic strength 0.075, 16 hours at 3.0 ma) was carried out on serum taken from rhesus and Cercopithecus sp. monkeys at various intervals following exposure to graduated numbers of S. mansoni cercariae. Both types of monkeys showed a similar total protein and A/G ratio until about the 61st day of infection when a sharp increase in percentage of globulin occurred, with a corresponding decrease in albumen. Study is being continued so as to cover the complete course of infection.

Summary:

No change from the normal occurs until about the 61st day of infection when there is a sharp increase in percentage of globulin and a corresponding decrease in albumen.

Study 12. Studies on schistosomiasis in Cercopithecus monkeys: Comparison of the development of CHR and COP antibodies in Cercopithecus and Macaca mulatta.

Progress:

Three groups of monkeys of each species were exposed to S. mansoni cercariae according to the following plan: Group I, single exposure to 200 cercariae; Group II, single exposure to 50 cercariae; Group III, multiple exposures to 10 cercariae at 40-day intervals. Sera are being collected at varying intervals and tested for COP and CHR reactivity for the purpose of comparing the development of reactivity according to species of host and magnitude of exposure.

Group I monkeys were tested 1, 2, 3, 5, 8, 11, and 14 weeks after exposure. Both species were negative for COP and CHR through 5 weeks, except for one M. mulatta which gave a 4+ CHR in the 2nd week; COP was negative at this time, however. All monkeys gave 4+ CHR's by the 8th week. Positive COP's were also observed at this time, but the extent of reaction varied in the 2 species; for example, all Cercopithecus sera reacted with a greater number of eggs than did M. mulatta sera, 21-31% vs 12-23%. At 11 weeks CHR reactivity was still high for all monkeys, while COP readings dropped in Cercopithecus (5-29%) and increased in M. mulatta (25-26%).

Groups II and III were tested at 1, 3, 6, and 14 weeks after exposure. All monkeys were negative for both procedures at 1, 3, and 6 weeks. At 14 weeks all gave 4+ CHR's and COP reaction was in the range of 22-36% of eggs.

The first antigenic stimulus in these monkeys was provided by cercariae. However, with the schedule of bleedings used, CHR antibodies were not detected earlier than COP antibodies except in one Macaca. The number of cercariae used in the exposures did not appear to have any bearing on the extent of either reaction or on time of the first appearance of the antibodies. Eggs were first detectable on the 35th to 43rd day of infection. By the 56th day COP reactions were demonstrable, regardless of the size of the exposure. The increase in COP reactivity in M. mulatta at 11 weeks and the decrease in Cercopithecus was not related to the number of eggs passed at that time or previously. The mean number of eggs was markedly higher for Cercopithecus in all time intervals.

Summary:

CHR and COP reactivity developed at about the same time in both species of monkeys and independently of the magnitude of exposure received by the animals.

Study 13. Studies on schistosomiasis in Cercopithecus monkeys: A comparison of the immunologic response to Co^{60}-irradiated cercariae in Cercopithecus and M. mulatta monkeys.

Progress:

Thus far, investigations on immunization of animals to schistosomiasis by exposure to irradiated cercariae have been done with mice and the rhesus monkey. Since the green monkey (Cercopithecus sp.) differs from the rhesus in other aspects to its response to S. mansoni, it was desirable to compare the immunologic response elicited by irradiated cercariae in the two species. The study as planned will run considerably over a year. At this time the animals are still in the process of being immunized, hence this report is limited to observations made to date.

Four green monkeys from St. Kitts Island (BWI), 4 green monkeys from West Africa, and 3 rhesus monkeys have been exposed on 3 occasions at approximately 40-day intervals to 3000 S. mansoni cercariae exposed to 3000 rads from a Co^{60} source. A fourth exposure is to be given. At the time of each exposure a group of mice are exposed to normal cercariae and another group is exposed to irradiated cercariae. These serve as infection controls. Mice that received normal cercariae at the time of the first exposure had a yield of 52.3% adult worms; the second group receiving normal cercariae had a yield of 39.4%. The third group is to be necropsied at a later date. Mice that receive irradiated cercariae are to be processed according to their own protocol.

Only 1 monkey, a St. Kitts green, has shown a skin reaction in the area of the exposure after each of the 3 exposures. Three others, 1 St. Kitts and 2 West African green, exhibited a skin reaction after the 3rd exposure only. No skin reaction has been seen in any of the rhesus monkeys.

Beginning on the 45th day of the experiment, all monkeys have had stool examinations twice weekly with the FE-BA concentration technique. All examinations have been negative for S. mansoni ova.

Monkeys are bled according to a schedule and various serologic and immunologic tests will be done on the sera. Up to the present, 2 of 4 St. Kitts greens, 2 of 4 West African greens, and all 3 rhesus exhibited 4+ CHR's by the 43rd day after the initial exposure; 1 more St. Kitts and 2 additional West African greens became 4+ CHR positive by the 92nd day. The remaining St. Kitts green have stayed CHR negative, but its serum has a cidal effect on cercariae. The COP test has also been performed on the sera. The 3 rhesus and 1 of the West African greens exhibited weak reactivity by the 92nd day of the experiment; all other sera have been negative.

Summary:

Preliminary results of a long-term experiment show that all but 1 of green monkeys (Cercopithecus sp) and all of 3 rhesus monkeys exhibited 4+ CHR reaction not later than the 92nd day after the first exposure and about 50 days after the second exposure to irradiated cercariae. The rhesus have exhibited weakly positive COP tests but only 1 green monkey has so reacted. Repeated stool examinations have remained negative for S. mansoni ova.

Study 14. Studies on schistosomiasis in Cercopithecus monkeys: Comparison of immunoprecipitins in sera of Cercopithecus and Macaca mulatta during the course of infection.

Progress:

Three monkeys of each species are being compared as to formation of precipitating antibody by immunodiffusion and microimmunoelectrophoresis following exposure to 200 cercariae. All antigens are prepared in 0.85% saline at 1:25 dilution (dry wt/vol) with homogenization and sonication. Preliminary observations from immunodiffusion tests show the following: (1) After 35 days of infection sera from 1 Cercopithecus reacted against an egg antigen but not against cercarial or adult antigens. None of the other monkeys reacted against any of the antigens. (2) After 60 days all 3 Cercopithecus reacted with egg antigen (with 2, 3, and 3 bands, respectively) as did the Macaca (3, 3, 1 bands). With adult antigen, sera from only 2 monkeys reacted, each serum producing 1 band. Against cercarial antigen, 2 of the Cercopithecus produced 1 and 2 bands; only 1 Macaca reacted with cercarial antigen, producing 2 bands. (3) All sera tested 77 days after exposure reacted against all 3 antigens. With cercarial antigen, Cercopithecus showed 1, 2, and 1 bands, and Macaca showed 1, 1, and 2 bands. Cercopithecus exhibited 1, 2, and 2 bands against adult antigen, while Macaca gave 1, 1, and 2. Two of the 3 monkeys of each species reacted against egg antigen, giving at least 2 bands each except one of the Macaca which gave 3 bands.

Tests on the identity of bands produced with 77-day sera were performed on Ouchterlony plates according to a procedure that would determine if coalescence occurred or not. A high degree of similarity between the 2 species in regard to immunoprecipitin formation was demonstrated by the extent of coalescence of bands (reaction of identity). At least one band coalesced with all others when tested among and between the species with all 3 antigens.

Studies on the comparison of the electro-mobility of immunoprecipitates are in progress; the method of microimmunoelectrophoresis described by Scheidegger is being used for this purpose. Comparisons will also be made between monkey and human infections and with artificially immunized rabbits.

Summary:

Preliminary observations by immunodiffusion have shown that development of immunoprecipitins occurred between 35 and 77 days of infection. Other observations indicate a high degree of similarity between the 2 species in respect to immunoprecipitin formation.

Study 15. Studies on schistosomiasis in Cercopithecus monkeys: Comparison of immunoprecipitins in sera of Cercopithecus and Macaca mulatta after exposure to irradiated cercariae of S. mansoni.

Progress:

Eight Cercopithecus sp. and 3 M. mulatta monkeys were exposed on 3 occasions at approximately 40-day intervals to 3000 cercariae treated with 3000 rad in a Co^{60} source. A 4th exposure is scheduled. The formation of precipitating antibody in the 2 species is being compared by immunodiffusion and microimmunoelectrophoretic methods.

A strong antibody response against a cercarial antigen was evident in sera taken 101 days after the 1st exposure, or about 40 days after the 2nd exposure. Two Cercopithecus produced 1 band; 4 others showed 2 bands; and the remaining 2 produced 3 bands. The 3 M. mulatta produced 2 bands each. The bands in M. mulatta gave a reaction of identity with those in Cercopithecus. There was also coalescence between 2 bands in Cercopithecus and in rabbits which had been immunized with a cercarial antigen.

The response against an adult antigen in 101-day sera was far less dramatic: Only 1 Cercopithecus serum reacted and it produced 2 bands. Of these, 1 coalesced with the 1 band formed in only 1 of the M. mulatta sera. Thus, only 1 of each species reacted against adult antigen.

None of the sera reacted against an antigen prepared from eggs.

Studies now in progress, but for which results are not yet available, are concerned with a comparison between immunoprecipitin development in monkeys with normal infection and those exposed to irradiated cercariae.

and between these and rabbits immunized with adult and cercarial antigens.

Summary:

Preliminary observations from immunodiffusion tests show that both species exhibit a strong response against a cercarial antigen with 101-day sera, while little or no reaction was exhibited against adult and egg antigen.

Study 16. The serologic response of intraperitoneally implanted cercariae of S. mansoni.

Progress:

Work done elsewhere has showed that profound changes occur in the response of cercariae to certain types of sera and their compatibility with water and saline immediately following skin penetration. Since these changes seemed to be related to the act of penetration, it is of interest to determine if the same phenomena occur in developing forms which had no opportunity to penetrate skin. For this study S. mansoni cercariae were injected IP into mice and then removed after varying periods of time, following which they were exposed to various sera and water. The age of the forms is taken as the time between injection and recovery. The experiment is not complete but the following observations occur regularly: (1) forms $2\frac{1}{4}$ - $2\frac{1}{2}$ hrs. old or more no longer show CHR activity in immune human serum; (2) the cidal reaction in normal rat serum is lost at the same time; (3) forms 30 minutes to 1 hour old no longer exhibit CHR in immune mouse serum, instead, a cidal reaction occurs that persists until forms are $2\frac{1}{2}$ hours old, after which it also disappears; and (4) peritoneal forms up to 120 hours in age seem to be equally well adapted to saline and to water, at least through 4 hours observation. Work is now in progress to associate these observations with changes in the penetration glands of cercariae and stages immediately following.

Summary:

Specific responses of cercariae to certain sera disappear after about $2\frac{1}{2}$ hours' contact with mouse peritoneum.

Study 17. The antigens of Schistosoma mansoni: Comparison of the schistosomule with adult and cercarial forms by immunoprecipitin analysis.

Progress:

In the development of a program on the immunology of S. mansoni, work was started in the last half of the reporting period on the antigenic characterization of 5-day old schistosomules. These are produced by injecting massive numbers of cercariae into the peritoneal cavity of mice and then recovering them at the desired time. To date, rabbits have been immunized with an antigen prepared from such forms; other

rabbits have been immunized with adult and cercarial antigens. All antigens have been used with Freund's complete adjuvant. Time has not permitted comparison of all antisera and antigens, therefore, the observations reported here are to be considered preliminary in nature.

Adult antigen vs anti-adult sera produces 4 bands by double diffusion (dd); 4 bands are also given by immunoelectrophoresis (IEP). Adult antigen vs anti-schistosomule serum gives 1 band by dd that separates into at least 4 by IEP. It has not been determined if all or any of these are the same as those seen in an adult antigen vs anti-adult system.

Cercarial antigen vs anti-adult serum gives as many as 9 bands by IEP. Against anti-schistosomule serum, cercarial antigen gives at least 2 bands by dd and 3 by IEP.

It has not yet been possible to test a schistosomule antigen against homologous or heterologous antisera.

The discrepancy between the number of bands found in dd and IEP has been studied preliminarily by serial dilution of the antigen. In the adult antigen vs anti-schistosomule serum system, the 4 bands given by IEP with antigen diluted 1:25 remained the same when antigen was diluted to 1:100 and 1:200, although the bands diminished in intensity with dilution. However, with dd 1 thick band formed with 1:25 antigen; this band resolved into 2 with antigen at 1:50, but at 1:200 4 bands were present, one of which was distinctly different from all the others. Of these 4 bands, 2 definitely coalesced with bands given with lower dilutions of antigen. Thus it would seem that the concentration of the antigen used in double diffusion tests should be carefully controlled.

Summary:

Preliminary observations indicate that 5-day schistosomules differ antigenically from both cercariae and adults. Other findings suggest that concentration of antigen in double diffusion can be a critical factor in determining the number of precipitin bands that may result.

Study 18. Observations on the development of acquired resistance to Schistosoma mansoni in new-born rats.

Progress:

Sadun and Bruce (Exp. Parasit. 15 (1): 32-43. 1964) have reported that acquired resistance to S. mansoni was elicited by injections of worm homogenates in rats which were only 1 or 14 days old at the start of the experiments. Rats of these ages also developed a significant level of resistance after a prior infection. Earlier observations in our laboratory indicated that prior infection of new-born mice resulted in "immunological tolerance" of a subsequent exposure. Therefore, it was considered desirable to repeat in part the experiment with rats for the purpose of

confirming earlier observations and to determine the effect of the route of exposure on the development of resistance in new-born rats.

One to 3-day old rats were exposed to about 200 S. mansoni cercariae by either the IP or the PC route. Development of resistance was assayed by the response to a challenge exposure with 950 - 1000 cercariae made four weeks later by the homologous route. Appropriate controls were incorporated. Necropsies were made 4 weeks after the challenge exposure. Two replicates were done with both routes.

Negligible resistance was conferred by IP exposure. A 13% reduction in challenge infection worm burden was observed in one replicate and only 6.5% in the other. However, with PC exposures there was 42% reduction in the first replicate and 80% in the second.

It cannot be concluded from this experiment that prior infection conferred a significant level of resistance in new-born rats. However, results indicate that such immunized rats did not respond to challenge as though it were a primary exposure. Furthermore, the degree of response to challenge seems to be directly related to the magnitude of the immunizing worm burden at the time of challenge. This, in turn, is associated with the route of exposure. The PC route would be preferred since it consistently gave appreciably higher yields than did the IP route.

Summary:

Unequivocal evidence for the development of resistance was not observed. However, it was apparent that rats which had been administered a prior infection by the percutaneous route did not respond to challenge as through it were their first exposure.

Study 19. Infectivity of Schistosoma mansoni cercariae from Australorbis glabratus maintained on different diets.

Progress:

A group of A. glabratus was maintained solely on each of the following foods: the standard formula (containing Cerophyl, wheat germ, Glandex, and powdered milk; without alginate), romaine lettuce, boiled, dried malanga stem, squash, Gaines Meal dog food, and a chicken food. In each case the snails were cultured in 10-gallon aquaria in static water. Mice were exposed to cercariae from each group of snails on 4 or 5 occasions. Whenever exposures were made, cercariae used routinely in exposing animals were included as "controls".

Worm recovery rates varied little for cercariae for snails fed the several types of food. The range of variation was from 43 to 61% for the standard formula and 42 to 61% for the "control" exposures. Generally the worm recovery rate for cercariae from other snail-foods fell within the above ranges with comparable fluctuations. The highest worm recovery

rate for any food was 72%, for cercariae from snails fed malanga stems.

Although the infectivity of cercariae was relatively uniform for all foods, the numbers of cercariae emerging from snails was low for those fed with squash and malanga. This may have been due to the small size of the snails on this diet.

There was less variation in the worm recovery rates for cercariae collected on the same day from different foods than for cercariae collected on different days from the same snail-food. For 4 out of 9 days of exposure the worm recovery rate for cercariae from different foods were as follows: 61, 61, 61; 43, 47, 44, 45; 43, 45, 43, 47, 46, 42; and 54, 52, 55, 49, 62. On the other 5 days the similarity of figures was somewhat less, but yet the variations were not as great as those shown for the same food on different ways. The observations here noted could be due to physiological variations in the snails from time to time, as indicated by Evans and Stirewalt (1961, Exp. Parasitol., 1: 19); however, with 7 different lots of snails it is unlikely that physiological variations could be synchronized to occur on the same days. It appears, then, that technical irregularities in handling cercariae should be explored as a possible cause of variation in cercarial infectivity.

Summary:

A group of Australorbis was maintained solely on each of the following foods: standard formula, lettuce, malanga stem, squash, Gaines Meal dog food, and a chicken food. Cercariae from infections in each group of snails were found to be about equally infective with worm recovery rates ranging from about 42 to 61% for 4-5 exposures made on different days. In contrast, there was less variation than this for worm recovery rates from cercariae collected on the same day from different foods. It is speculated that this difference may be related to some unrecognized technical irregularity.

Study 20. The effects prolonged low concentrations of copper sulfate on the emergence of Schistosoma mansoni cercariae from Australorbis glabratus.

Progress:

Copper sulfate at 0.25 ppm was lethal to all lab-reared stages or sizes of hatched Australorbis glabratus after 1 week of exposure, the newly hatched being most susceptible and the 8-10 mm specimens the most resistant. With a concentration of 0.1 ppm, hatching of eggs was reduced by about 50%.

Infected snails did not survive for more than 1 week in 0.1 ppm of $CuSo_4$. During this interval the cercarial output declined from 1600 per snail on the 1st day to 100 on the 4th day, yet during this period only about 50% of the snails had succumbed. In 0.05 ppm $CuSo_4$, the infected snails generally survived for as long as a month. During this interval

the cercarial emergence declined to near zero after 2-3 weeks in several replicates. An attempt is being made to determine whether the cercarial output will be renewed after treatment of snails is stopped.

Summary:

This project has been continued through 1964-65 confirming the observations that copper sulfate at 0.05 ppm suppresses cercarial emergence within 2-3 weeks, that 0.1 ppm killed all infected snails within 1 week, and that about 0.25 ppm will kill mature, uninfected, laboratory-reared snails within one week.

Publications:

1. Enhancement of Acquired Resistance Against Schistosoma mansoni in Albino Mice by Intraperitoneal Immunizing Exposures. Lyman P. Frick, Lawrence S. Ritchie, Wilda B. Knight, and Joseph H. Taubr. Journal of Parasitology 51: 230-234, 1965.

2. Urinary Protein Alteration in Experimental Schistosomiasis mansoni. Seymour Garson, Elsa Agnoli, and Lyman P. Frick. Journal of Parasitology 51 (No. 2, Sec 2): 43, 1965.

3. Effect of Fuadin Therapy on the Circumoval Precipitin (COP) Test in Mice Infected with Schistosoma mansoni. Seymour Garson, J. Oliver-Gonzalez, and Elsa Agnoli. Journal of Parasitology 51 (No. 2, Sec 2): 63, 1965.

4. Problem Areas in Experimentation on Acquired Resistance Against Schistosomiasis. Lyman P. Frick, Lawrence S. Ritchie, and Wilda B. Knight. Journal of Parasitology 50 (No. 3, Sec 2): 22, 1964.

5. Biology of Schistosoma mansoni in the Green Monkey (Cercopithecus) Occurring on St. Kitts Island (British West Indies). Lawrence S. Ritchie, Wilda B. Knight, and Jose Oliver-Gonzalez. Journal of Parasitology 50 (No. 3, Sec 2): 21-22, 1964.

6. Effects of Low Concentrations of Copper Sulfate on Emergence of Cercariae of Schistosoma mansoni from Australorbis glabratus. Lawrence S. Ritchie, Luis A. Berrios-Duran, and Rogelio Sierra. Journal of Parasitology 51 (No. 2, Sec 2): 31-32, 1965.

7. The influence of infection intensity of Schistosoma mansoni on resistance against existing and subsequent infections in Macaca mulatta monkeys. Lawrence S. Ritchie, Wilda B. Knight, Donald B. McMullen, and Franz vor Lichtenberg. American Journal of Tropical Medicine and Hygiene (In press).

RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6401	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
1 07 65	A. New	U / U	"NA"	NL	A. Work Unit

10a CURRENT NUMBER/CODE: 61145011 3A014501B71P 10 031

10b PRIOR NUMBER CODE: 61145011 3A014501B71P 11 02

11 TITLE

(U) Ecology and Control of Intermediate Hosts of Schistosomiasis and Other Parasitoses (PR)

12 SCIENTIFIC OR TECH. AREA	13 START DATE	14 CRIT. COMPL. DATE	15 FUNDING AGENCY
002600 Biology	06 61	"NA"	Other | DA

16 PROCURE. METHOD	17 CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18 RESOURCES EST.	a. PROFESSIONAL MAN YEARS	b. FUNDS (In thousands)
C	"NA"	PRIOR FY 65	2	55
		CURRENT FY 66	1	19

19 GOV'T LAB INSTALLATION ACTIVITY

NAME / ADDRESS: Hqs US Army Medical Research & Dev Comd
Washington, D. C. 20315

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20 PERFORMING ORGANIZATION

NAME / ADDRESS: U. S. Army Tropical Research Med. Lab.
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21 TECHNOLOGY UTILIZATION	22 COORDINATION
"NA"	"NA"

23 KEYWORDS

Schistosomiasis, Australorbis glabratus, vector, molluscicide

24 (U) Tech Objective - It is currently believed that destruction of the snail vectors is an essential means of eradicating schistosomiasis. This may be accomplished by molluscicides or possibly by biological agents, e.g., competitor snails or ecological influences. Knowledge of the vector's life history and enemies, and of the deleterious effects of natural influences, is essential.

25 (U) Approach - A large program devoted to laboratory screening and comprehensive evaluation of molluscicides against Australorbis glabratus is being pursued in cooperation with the WHO. Laboratory screening tests have been made on about 60 products, of which 12 have been subjected to comprehensive testing in the laboratory and field.

(U) Progress - Time-concentration relationship studies show that some compounds are equally effective in 1-, 6-, or 24-hour exposures. Others are better in one exposure or another. Stability of chemical working solutions range from a few hours to several months. Copper sulfate has advantages in prolonged concentrations. Susceptibility of
26 the snail in a stage-size array varies from one chemical to another. NaPCP, ICI-24223 and WL 8008 are detoxified by sunlight but not Bayluscide, Dinex or organo-tins. A Test to detect adsorption is being developed. Several compounds have been evaluated in a new field screening test. Test of Bayluscide in multiple applications in progress.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
"NA"	"NA"

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST. FUNDS (In thousands) CFY+1	36

DD FORM 1 AUG 64 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A014501B71P:	Basic Research in Support of Military Medicine
Task No. 3A014501B71P 11:	Zoological Sciences
Work Unit No. B71P 11 02:	Ecology and Control of Intermediate Hosts of Schistosomiasis and Other Parasitoses (PR)
Reporting Installation:	U. S. Army Tropical Research Medical Laboratory APO New York 09851
Division:	Medical Zoology
Period Covered by Report:	1 July 1964 - 30 June 1965
Professional Authors:	Lawrence S. Ritchie, PhD Lyman P. Frick, Colonel, MSC
Technical Assistants:	Luis A. Berrios Duran, BA; Wilma Q. de Jimenez, BA; Rogelio Sierra; Henry Wessel
Collaborators:	Irving Fox, PhD, School of Medicine, UPR
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

BODY OF REPORT

Project No. 3A014501B71P: Basic Research in Support of Military Medicine

Task No. 3A014501B71P 11: Zoological Sciences

Work Unit No. B71P 11 02: Ecology and Control of Intermediate Hosts of Schistosomiasis and Other Parasitoses (PR)

Study 1. Definitive laboratory screening of candidate molluscicides.

Progress:

Chemical companies providing compounds:

- Chapman Chemical Company
- M&T Chemicals
- Shell Chemical Company
- Stauffer Chemical Company
- Ben Venue Laboratories
- U. S. Army Natick Laboratory
- Fission Overseas Ltd.

Results of screening: The Chapman Chemical Company has admitt products, of which a majority were active in the range of 10 ppm; two were effective at 1 ppm, including tetra- and pentachlorophenol . Especially the latter is deemed to be of special interest because formly it was set aside in favor of its more soluble sodium salt (NaPCP). Now, solubility of molluscicides is not rated as important as previously, and PCP is more toxic than its sodium salt.

Three organotin compounds provided by M&T Chemicals showed good activity in the range 0.1 to 0.4 ppm.

The Natick Laboratory of the U. S. Army submitted two organolead compounds, but they were active only at 10 ppm, which is not as good as several compounds of this type previously submitted by the same laboratory.

Fission Overseas Ltd. provided two compounds that were active at 1 ppm or less.

The Shell Chemical product, WL 8008 (18% concentrate), gave an LC_{90} of .06 ppm.

Two products from Stauffer Chemical Company were highly active at 0.1 ppm (LC_{90}). This company has expressed interest in having these products included for comprehensive evaluations.

An extensive series of substituted nitrosalicylanalides submitted by Ben Venue Laboratories have proved interesting and eight have been active at 2 ppm or less, whereas the rest were mostly active in the range of 10 ppm. Of the better ones two have shown good activity at 0.25 ppm. These can be recognized in the table as 343 NCS (B) and 33 NIS. They are not quite as effective as Bayluscide.

Two companies have provided chemicals for definitive screening for the first time in 1965. These include Monsanto Chemical Company and Dow Chemical Company. Representatives of American Cyanamid and Pitman-Moore Company both indicated that they will probably be submitting compounds for evaluation in the near future.

Summary:

Five chemical companies submitted products for definitive laboratory screening. The test includes a ten-fold dilution series of 0.1, 1.0 and 10 ppm with 24-hour exposures. Of special interest was a series of 43 substituted nitrosalicylanalides submitted by Ben Venue Laboratories. These products are homologues of the active ingredient of Bayluscide and about 12 of them were active within the range of 0.1 to 1.0 ppm. Some of these and a few others from different companies warrant further consideration with comprehensive evaluations.

Study 2. Molluscicidal time-concentration relationships.

Progress:

The procedure currently used in our laboratory for this evaluation involves determining a dilution series for 24-, 6-, and 1-hour exposures with mature (13-15 mm) snails. Previously, a time gradient from 3-24 hours (3, 6, 12 and 24 hours) were used with a single concentration, LC_{90} value (WHO Mol/Inf Series, 1962, No. 7, 7-9). This change was initiated when it was found that some molluscicides are effective with exposure intervals of 1 hour or less (Bayluscide, Moluscid and WL-8008), while others are relatively ineffective below 6 hrs. and some require 24 hours exposures for maximum results. Now we recognize that both plans of evaluation may be warranted for superior molluscicides. Compounds tested with 24-, 6- and 1-hour exposures in dilution series include six organotins, 4 dinitro compounds, WL-8008 (Tritylmorpholine), and several substituted nitrosalicylanalides.

Organotin compounds: Among six organotin compounds tested, tri-n-propyl tin oxide, tri-n-butyl tin acetate, tri-butyl tin oxide and butyl tin oxide showed great activity at 0.1 ppm in 24-hour exposures. On the basis of toxicity alone, then, they could be classified with Bayluscide. These same compounds were also effective in 6-hour exposures since the amount of chemical required for this period was not disproportionately greater than that required for 24-hour exposures. None of the compounds could be considered effective in 1-hour exposures, because too great a

chemical concentration was required. The organotin compounds appear to warrant prolonged, low concentration tests (Ritchie et. al. (1964) Bull. Wld Hlth Org. 31, 147).

Dinitro-compounds: For four (4) dinitro-compounds tested, the order of effectiveness with 24-hour exposures was dinitro-o-cyclohexylphenol, dinitro-o-tertbutylphenol, dinitro-o-secbutylphenol, and dinitrophenylphenol, LC_{90} values being 0.55, 0.74, 1.25 and 4.1 ppm, respectively. This relationship changed somewhat with 6-hour exposures, but dinitro-o-cyclohexylphenol remained decidedly better than the others. None of them were effective with 1-hour exposures.

WL-8008 (Trityl morpholine): This product proved relatively uniform in effectiveness with 24-, 6-, 4-, 2-, and 1-hour exposures. The respective LC_{90} time-concentration values with mature snails being 1.49, .58, 1.5, 1.4, and 0.5. The corresponding values giving 100% mortalities were, 2.4, 1.2, 1.3, greater than 1.0, and 1.0. Whether the higher level of efficiency obtained with 6-hour and 1-hour exposures is significantly different is a matter of interest. However, it is important that the efficiency index is relatively uniform for all exposure intervals from 24 down to 1 hour (WHO Mol/Inf Series, 18, (VIII), 1964).

Substituted nitrosalicylanalide: Among 43 substituted nitrosalicylanalides submitted by the Ben Venue Laboratories, several have been evaluated on a basis of a time-concentration relationship. Two had mortality end points with 1 ppm and 2 with 0.5 ppm in 24-hour exposures. Three were relatively effective with 6-hour exposures, while only two could be considered as effective with 1-hour exposures.

Summary:

Four dinitro-compounds were tested in dilution series with 24-, 6-, and 1-hour exposures. One of these, long known for its molluscicidal activity (dinitro-o-cyclohexylphenol) was superior to the other three. WL-8008 (tritylmorpholine) was uniformly effective at 24-, 6-, 2-, and 1-hour exposures. Among four substituted nitrosalicylanalides, three were effective with 6-hour exposures and two with 1-hour exposures.

Study 3. Chemical stability of molluscicidal compounds in working dilutions (LC_{90}).

Progress:

The objective of this investigation was to determine the chemical stability of molluscicidal compounds in working dilutions. Solutions in litre quantities with previously established LC_{90} values for 24-hour exposures were allowed to stand for varying times, ranging from 0 hours to 32 days, after which snails were added for 24-hour exposures. Thereafter a recovery period of 24 hours was allowed before mortalities were recorded.

NaPCP at 2 ppm did not lose activity through 32 days. Dinex, with a concentration of 0.8 ppm, lost activity between 16 and 32 days. Bayluscide showed minimal decline in stability through 6 days, but was inactive after the 7th day. The several forms of ICI-24223 lost activity completely after 2 days, with partial loss after 1 day. Triphenyllead chloride and triphenyllead acetate were stable for 4 days, but have not been tested for longer intervals. Tri-n-butyltin oxide is stable for 4 days at 0.1 ppm, while 0.2 ppm was stable for as long as 64 days. Tri-n-propyltin oxide showed some instability after 2 days and 4 days with 0.1 ppm, but was stable for 64 days at 0.2 ppm. Two chemicals from each, Fission Ltd. and Stauffer Chemical, were stable after 4 days and are awaiting tests with longer periods of standing. It is especially interesting to find that copper sulfate at 0.4 ppm was stable for as long as 4 days; longer standing periods remain to be tested.

There is no criterion at present as to how stable a compound must be in working dilutions in order to afford maximum efficiency under field conditions. This may well vary with methods of application and the type of habitat that is being treated. It is speculated that static water might benefit from the use of chemicals that have marked stability, whereas in flowing water a 4-day interval might be somewhere nearer the critical point. However, we have had good success with ICI-24223 (Moluscid formulation) for distances of 3/4 and 1½ miles in flowing water at a concentration of 3 ppm with 2-hour exposures. In the circumstances of the above test the flow rates were such that the chemical could perform within a 24-hour period. Therefore, an arbitrary 4-day stability period may be too high.

Summary:

The stability of working concentrations (LC_{90} values) of 15 compounds has been determined by testing against snails after varying periods up to 32 days. One product (ICI-24223) began to deteriorate within 24 hours and was totally inactive after 48 hours. Most were active through 4 days, while NaPCP and Dinex were active for over 16 days.

Study 4. Detoxification of Bayluscide under storage.

Progress:

The objective of this study was to find out whether the toxicity of Bayluscide is reduced under storage. Samples received in 1961, 1963 and 1964 were available for comparison in the latter part of 1964. Snails of 3 different ages (less than 2 months, 2-3 months, and greater than 3 months) for 3 different lab-reared stocks and field snails from one locality were used. Lab-reared snails from different conditions of culture in 2 laboratories were available for testing.

There was well-defined evidence of loss in toxicity after 3 years of storage, and a suggestion of minimal deterioration after one year. The 2

older age groups showed this more clearly than the youngest category (6 weeks). Regardless, even after 3 years the molluscicide still equalled the claim of the manufacture, that 0.5 ppm affords 100% mortality, and doubling the amount of the 3-year old chemical equalled the activity of fresh material.

The results revealed that young-mature snails (6 weeks) are more susceptible than older specimens (2-3 months and greater than 3 months); there was minimal difference in susceptibility of the 2 older groups. Two strains of snails obtained from the same field source, one an albino, and cultured similarly (static water, aerated) were about equally susceptible. The third laboratory strain, reared in continuously flowing water was a little more susceptible. Field snails required about twice as much Bayluscide to give 100% mortalities as compared with lab-reared stocks.

Summary:

This compound was found to lose activity to a limited degree within one year and considerable activity within 3 years. However, even after 3 years it equalled the claim of the manufacturer that 0.5 ppm affords 100% mortality, and doubling the amount of the 3-year old chemical equalled the activity of fresh material.

Study 5. Activity of WL-8008 against a stage-size array of Australorbis glabratus.

Progress:

Because this molluscicide is highly active even with short-interval application, we chose to use 6- and 1-hour exposures for this comprehensive evaluation. As previously observed by other investigators, we found that eggs in all stages of incubation were highly resistant to WL-8008. The juvenile (3-5 mm) stage was most susceptible. In contrast, newly-hatched snails were as resistant, if not more so, than any other of the hatched stages. This seemed to be more true with 6-hour exposures than with the 1-hour exposures. Snails that were 8-10 mm (adolescent) were more resistant than either the 3-5 and 13-15 mm specimens.

Summary:

This compound was tested in 6- and 1-hour exposures against eggs newly-hatched, juveniles, adolescents, and mature snails. Activity was negligible against eggs, and the newly-hatched snails were as resistant, if not more so, as any other of the hatched stages.

Study 6. Effects of sunlight on molluscicides.

Progress:

Various molluscicides were prepared in liter volumes of water using

LC_{90}-LC values for 24-hour exposures. The containers were then exposed to full sunlight for 1, 2 and 4 hours (an incident reading of 500-700 was obtained with the Weston meter) and snails were then placed in test for 24 hours. The activities of Bayluscide, Dinex and tri-n-butyl tin acetate were not affected by 4 hours exposure to strong sunlight. An organolead compound was moderately stable, while the activity of ICI-24223 and NaPCP was considerably reduced in 1 hour. In this test NaPCP can serve as a standard, since the mortality drops from 100% to 50% after 1 hour exposure to sunlight. By this means results from one test to another can be compared.

Summary:

The activity of ICI-24223 in all its forms, and NaPCP are considerably reduced by 1-hour exposure to sunlight. Bayluscide, Dinex and tri-n-butyl tin acetate were not affected by 4-hour exposures to strong sunlight.

Study 7. The detoxifying action of certain materials against molluscicides.

Progress:

Comments are commonly made in the literature to the effect that molluscicides are inactivated by various materials, using the word "absorption" as a means of explanation. It appears that very little is known about the effects of the various materials that occur in natural waters, including minerals of hardness, organic materials and particulate matter, against molluscicidal compounds. In an attempt to assay the effects of inorganic and organic materials, and particulate matters against molluscicides, beef extracts, feces of rats, and clay soil were used with several different molluscicides. In addition to the above materials we are also investigating the possible use of reagent grade powdered charcoal, and bentonite (a refined clay used serologically). The molluscicides thus far tested include sodium pentachlorophenate (NaPCP), Dinex, Bayluscide, ICI-24223, copper sulfate and cuprous oxide. Different concentrations of the detoxicants are prepared in litre quantities of water and the molluscicide is added in quantities equal to the LC_{90} value for 24-hour exposures. In a few cases it is apparent that we failed to repeat the toxicity of earlier testing.

It is apparent that NaPCP is not affected by beef extract, only a little by feces at 80-100 ppm, while clay soil had minimal if any effect against this molluscicide. Dinex was not affected by beef extract at 40 ppm or rat feces in a concentration of 100 ppm. Clay soil, however, appeared to have reduced the toxicity of this molluscicide. Bayluscide showed some decline in mortality both with rat feces at 100 ppm and soil, the reduction being in the order of LC_{90} to LC_{60}. ICI-24223 appears to be considerably more vulnerable to beef extract and the contents of rat feces, as compared with the above 3 molluscicides. However, it was not

ill-affected by soil. In fact, the base form of this molluscicide gave a higher kill with soil than without it. Copper sulfate was reduced in activity by the soil, but not particularly by the rat feces. Cuprous oxide was not affected by beef extract or rat feces at 3 ppm, but in a concentration of 1 ppm, which is usually lethal to all snails, complete detoxification occurred with 100 ppm of rat feces. The clay soil had negligible effect on this compound even at 1 ppm of the chemical.

The significance of these findings in relation to what happens in the field cannot be stated with confidence. Furthermore, additional laboratory testings must be done before a comparison can be made of the effects of the various materials on the several molluscicides. The relative stability of NaPCP against the detoxicants used is impressive in light of its vulnerability to sunlight. The difference between NaPCP and Dinex does not appear to be great in this regard, except that the latter is not vulnerable to sunlight. The suggested increase in toxicity of ICI-24223 with soil is worthy of further consideration. Of special interest is the minimal effect of rat feces on copper sulfate, in contrast to decline in activity due to soil.

Summary:

An assay was made on the effects of a beef extract, rat feces, soil, bentonite, and powdered charcoal against the activity of certain molluscicides. The detoxicants were prepared in litre quantities of water and tested against LC_{90} preparations of molluscicides in 24-hour exposures. It is believed that a series of materials can be identified which will indicate how molluscicides will perform under the effect of physico-chemical factors of the field environment.

Study 8. Susceptibility of lab-reared Helisoma duryi to molluscicides.

Progress:

Because of strict governmental quarantine regulations against Australorbis glabratus in United States, it was deemed appropriate to evaluate a non-vector snail for possible use in preliminary screening for molluscicides. The objective then was to provide a suitable non-vector snail in order to avoid the extra expense of maintaining a vector in a way that would preclude its escape into non-endemic habitats.

The non-vector snail used was Helisoma duryi collected in Puerto Rico by Dr. F. F. Ferguson. It was reared by us under laboratory conditions similar to those employed for Australorbis. Eight molluscicides were tested against Helisoma using the LC_{90} values that we had obtained in this laboratory for Australorbis. The snails were tested when they were 8-10 mm in diameter, which is a smaller size than we commonly use with Australorbis (13-15 mm). Two of the eight molluscicides tested (Bayluscide and ICI-24223) were also tested in a dilution series appropriate for computation of the LC_{50} and 90 values.

At least six of the eight molluscicides tested gave comparable results with Helisoma and A. glabratus, including NaPCP, Bayluscide, ICI-24223, cuprous oxide, tri-n-butyl tin oxide and tri-n-propyl tin oxide. The mortality obtained with Dinex was only 20% as compared with the expected 90%. Correspondingly, copper sulfate was a little less effective. Certainly none of the above molluscicides could have been missed in a preliminary screening procedure with Helisoma, since preliminary screening is usually carried out with a concentration in the range of 10 ppm, which is 5-100 times greater than the amount used in this evaluation. A dilution series of Bayluscide and ICI-24223 indicated that both are slightly less active against Helisoma than A. glabratus. ICI-24223 apparently is relatively less effective against Helisoma than Bayluscide, whereas against A. glabratus they give very similar results.

It appears that we have demonstrated a similarity in the molluscicidal activity of several compounds against Helisoma as compared with A. glabratus. The snails also cultured about equally well in the laboratory. If a chemical industry is confronted with the problem of added expense, due to quarantine restrictions by its government, certainly Helisoma can be recommended as an appropriate substitute for the vector snail in preliminary laboratory screening of molluscicides.

Summary:

This non-vector snail proved to have similar susceptibilities to molluscicides as Australorbis glabratus. This was found to be the case for 6 of 8 molluscicides tested. It is apparent from this study that the added cost incurred in rearing vector snails under strict government quarantine regulations can be eliminated by using a non-vector snail, Helisoma duryi.

Study 9. Evaluation of various foods for use in rearing Helisoma duryi snails.

Progress:

The objective of this study was to determine the relative adequacy of various foods for rearing snails, particularly commercial products for pets and laboratory animals, because they are readily available. The investigation is deemed significant in simplifying snail rearing, as a means of increasing interest in preliminary screening of molluscicidal compounds by chemical companies. Helisoma duryi snails were used since they had been shown in a previous study to have molluscicidal toxicities very similar to those of Australorbis glabratus.

The various foods used included commercial preparations for feeding monkeys, dogs, rabbits, and mice.* In addition, a standard formula

* Monkeys: Purina Monkey Chow -) Ralston Purina Co., St. Louis 2, Mo.
Rabbits: Purina Rabbit Chow -)
Dogs : Gaines Meal, Dog Food - General Foods Corp, White Plains, N.Y.
Mice : D&G Rat-Mouse Diet - The Price-Wolhoite Co., Frederick, Md.

(including cerophyl, wheat germ, glandex, and powdered milk, without alginate), and lettuce and squash separately, were tested. The latter was included because we had found it a good supplementary food; snails consume it readily, it decomposes relatively slowly, and it keeps especially long under refrigeration. The Australorbis used for comparison were reared in the same tank with the Helisoma.

All the foods gave similar growth rates, except squash and lettuce, for which they were considerably reduced.

There was considerable difference in egg output by Helisoma raised on the several foods. The largest number was obtained from snails fed the dog food, while monkey and mouse foods also gave good egg production. Squash and lettuce fed separately and without other foods were ineffective in relation to production of eggs.

Snails were tested for molluscicidal susceptibilities on two occasions when they were about 3 and 4 months of age. The first trial with 4 replicate tests of 10 snails each indicated snails fed either formula or squash were more resistant, but for the second trial (2 replicates) snails from all foods were about equally susceptible. Other possible factors that might affect molluscicidal susceptibilities have been investigated. The age of mature snails has been shown elsewhere in this report to affect results. Crowding does not seem to have much effect. Most resistant snails cultured have been some that were cultured in static water without change for considerable periods.

An excess of organic decomposition in aquaria due to the food is a problem in maintaining proper water quality. Continuously changing, or flowing water, alleviate this. With static water careful attention is necessary to avoid fouling. Aeration is an important factor in controlling this. The commercial foods used contained a binder which reduces the dispersion of the food substances throughout the water. It is believed that squash or lettuce should be used as a food supplement for any of the high protein commercial foods. By so doing, the amount of food with high protein content can be reduced, thereby protecting water quality, yet satisfying food demands of the snails. If large numbers of snails are not required, the volume of water per snail can be increased to assure optimal quality. This study and other cursory observations indicate that providing food for snails is not a problem as long as a well-balanced diet is made available, such as provided by many dry commercial pet or laboratory animal foods and supplemented either with lettuce or possibly squash. The suitability of a Helisoma snail has been clearly demonstrated for use in preliminary screening of molluscicides.

Summary:

To further simplify rearing of snails for preliminary screening of molluscicides compounds, a number of readily available commercial foods for lab-animals and pets was tested for suitability as snail food.

There was only a little difference in the results obtained with several foods, but the Gaines Meal dog food was particularly good. Chemical companies can conveniently maintain a snail stock at negligible cost for use in aquatic screening tests for "biological activity" which they routinely perform on new compounds.

Study 10. Attempts to improve snail culture to provide specimens that are as resistant to molluscicides as field snails.

Progress:

As shown in another section of this report, our lab-reared snails are more susceptible to molluscicides than field snails, the latter requiring about twice as much chemical to give mortalities equalling those for lab-reared specimens. Innumerable attempts have been made to increase the resistance of the lab-reared stock, but with little success, except the observation that maximum resistance against Bayluscide was not attained until an age of about 3 months. We have tried a variety of different foods and combinations, thereof, including lettuce, squash, boiled Malanga stem, several commercial dog and chick foods, and our standard formula (Cerophyl, wheat germ, glandex and powdered milk). Powdered alfalfa has proven equal to but no better than Cerophyl, in providing "bulk" in the formula. Resistance was not increased by any kind of diet. Crowding reduces growth rate of the snail, but we have seen no indication that this increases the susceptibility. Since our snail has been cultured for 8 years through 50 generations, a comparison was made with a first-generation stock from field collected snails. The new lab-reared was no more resistant to molluscicides than the old stock. The water quality, particularly the copper content, has been checked during the past year. This varies from 0.025 to .09 ppm and could be approaching a threshold of effect. Three (3) charcoal filters, 50 gal. drums filled with a mixture of charcoal and gravel, have been installed in the water line; however, this has not altered snail growth or susceptibility to molluscicides. The adequacy of our snail culture conditions, including continuously changing water, may best be indicated by survival of infected snails, which is about 4 months from time of exposure. During this patency period, the snails are subjected to conditions of "forced shedding" of cercariae twice a week.

Summary:

Innumerable attempts have been made to increase the resistance of lab-reared snails to molluscicides, but with little success. We have shown that mature snails continue to increase in resistance at least up to 3 months of age, but we do not have evidence of continued increase in resistance beyond this age. Factors found to have little effect included foods, crowding, and continued lab-culture up to 50 generations. The possibility of genetic selection of resistance is entertained.

Study 11. Detoxifying actions of the disposable polyethylene sack used as a liner for test containers.

Progress:

Several years ago this laboratory started using a polyethylene sack to line the litre paper buckets used for testing molluscicides. This was done and found appropriate because paper buckets were considered too expensive to discard after each test, while washing and breakage of beakers was considered too expensive and inconvenient, particularly in litre sizes such as we use as a standard in our testing program. Of immediate concern was whether the sacks in themselves might be toxic. This was not found to be the case. The next matter of concern was whether they might adsorb the chemicals. The latter does not occur with certain compounds, but does with others. Sodium pentachlorophenate, Dinex, copper sulfate, and Bayluscide are not adsorbed, while ICI-24223 and WL-8008 revealed detoxification when tested in the plastic sack liner. Of the two, WL-8008 seemed to be more affected than ICI-24223. A series of tests have been run with WL-8008 using 24-, 6-, and 1-hour exposures in three types of containers, including glass, wax-lined paper buckets and the latter lined with a plastic sack. The results were similar with the wax-lined paper buckets and glass beakers. Therefore, it appears appropriate to use the wax-lined buckets for chemicals that are adsorbed by plastic. It was found that detoxification was at a maximum when the exposures were for 24 hours, reducing with 6 hours, while with 1-hour exposures the loss in activity was negligible. It is concluded that tests concerned with detoxifying action of physico-chemical factors and any test of 24-hours duration should not be performed with the plastic sack. It is believed that the plastic liner can be used with regular toxicity tests involving 6- and 1-hour exposures.

It is also apparent that each new chemical that warrants comprehensive evaluations should be tested to determine adsorption tendencies in relation to the plastic. Whether there will be any correlation between adsorption by the plastic and under field conditions remains to be seen.

Summary:

As an inexpensive disposable test container, polyethylene sacks are routinely used to line litre containers. These did not prove toxic to snails, but apparently have a detoxifying effect on some molluscicides, but not others.

Study 12. A standardized "field-screening" test for molluscicides against aquatic snails in flowing water.

Progress:

A recent study group convened by WHO stressed the advantages of providing standardized "field-screening" tests for all types of snail

habitats. By definition this test should be limited to small habitats or limited volumes of water, in order to reduce to a minimum the cost of testing and the amount of chemical required. Such evaluation may be based on "planted" snails rather than natural population. This will allow for frequent testing and foster uniformity of results. Also control tests can be done periodically using the same habitat without chemical. The objectives of "field-screening" include: 1) confirmation of results of lab tests, 2) evaluation of different molluscicidal formulations and methods of application, 3) observation of toxicity levels for other animals and plants, and 4) detection of chemical dispersion.

This report describes a stream situation (El Toro) that appears favorable for field-screening candidate molluscicides. El Toro stream extends for about 1½ miles through Fort Buchanan, Puerto Rico. The first mile is a cement-lined ditch. Normal water flow is confined to a shallow trough along the upper limit (3/5 mile), while the water spreads over a broader surface downstream, giving a depth of about 1 inch, with a width of 3 to 4 feet. Under the latter conditions, the chemical is exposed to intense sunlight for a period of about ½ hour. The time of flow along the cement-lined ditch is about 1½ hours and the flow rate ranges from 400 to 1000 liters per minute. Water temperatures range from 25 to 35C at various times of the day and pH values are between 7.0 and 8.0.

Below the cement-lined ditch there is a typical unlined stream with dense marginal vegetation; but unfortunately only about 1/3 stream mile is appropriate for testing. At about mid-point along the usable mud-lined sector there is dilution from a sewage processing plant, emptying approximately 800 liters per minute, accounting for a high level or organic material for a distance of about 150 yards above the last exposure site. With other dilution points, there is about a doubling of the flow in the unlined test-sector as compared with the cement-lined section. There are six designated exposure sites throughout the entire test-sector of the stream, 3 in the cement-lined ditch and 3 in the unlined ditch.

Applications are made from constant head flow containers of different sizes. Liquid concentrates are applied without dilution in most cases, while wettable powders are mixed in stream water. Lab-reared snails are planted in either cages or baskets, using in most cases 20 snails per site.

Thus far only a few chemicals have been field-screened according to the above plans. Exposures have been mostly with 1 and 2 hours intervals. Initial testing is limited to the cement-lined ditch and for this 1, 2, and 3 to 4 ppm have been used with Bayluscide, Moluscid and WL-8008. With 1 ppm and 1-hour exposures all three compounds failed to kill at the lowest, or third exposure site. With 2 ppm efficiency was high at all three stations, and with 3 or 4 ppm all snails were killed even at site 3.

Control observations included, 1) testing a random sample of the snails in the laboratory, 2) the placing of snails above the point of application, and 3) return of the latter to the laboratory for testing.

Control snails, transported to and from the field and exposed to field conditions for the duration of the test, did not show increased susceptibility.

Chemicals that were tested throughout the entire distance of the cement-lined and unlined stream sectors included Bayluscide, Moluscid, and Dinex. In this case tests were mostly with 3 ppm and 2-hour applications. Doubling the exposure time was sufficient to compensate for dilutions in the unlined portion. With the exception of site 4, Bayluscide killed 100% of the snails at all 6 exposure points. Moluscid was effective through site 5, but completely failed at site 6. This lesser efficiency was overcome when the application was made at night, 100% of the snails then being killed at site 6. Dinex, with 1-hour application and 20 ppm was also effective through site 6 (100% mortality). Certainly, the time-concentration relationships were altered in the unlined stream sector, due to differences in flow-rate from middle to margins and from surface to bottom of the stream, which would tend to dilute the chemical but prolong exposure.

Summary:

Additional refinement has been made in the field-screening test described in the previous annual report. Considerable testing has now been done on Bayluscide, ICI-24223, WL-8008 and dinitro-o-cyclohexylphenol. The importance of this type of testing can now be documented from WHO reports, with the U. S. Army Tropical Research Medical Laboratory listed as one of several world centers where such testing can be done.

Study 13. Work projected for 1965-66.

1. Definitive laboratory screening: Several chemical companies have indicated they will have products to evaluate. This work will be carried out routinely.

2. Comprehensive laboratory evaluations: As highly molluscicidal compounds are identified, a battery of comprehensive evaluation tests will be imposed against each. A sequential plan of testing will be followed, including, 1) time-concentration relationships, 2) chemical stability, 3) stage-size array susceptibilities, 4) protective behavioristic traits of the snail against the molluscicide, and 5) tests on the effects of physico-chemical factors. If the results of the first two of the above tests are favorable, field-screening tests will be initiated as soon thereafter as the chemical is available. The other tests listed will be carried out while field-screening is underway.

Considerable time will be given to further standardization of comprehensive laboratory evaluations, particularly those associated with the effects of physico-chemical factors, particularly inorganic, organic and particulate materials.

3. Field-screening: These tests will be carried out according to the plan outlined in one of the above reports. Presently tri-n-butyltin oxide is under evaluation.

4. Field tests: a. A field test is being carried out with Bayluscide applying it repeatedly in a stream about 3 miles long that has numerous impoundments along its course. A drip system will not work in this situation and we are merely stirring the chemical into the water at the upper and lower levels of each impoundment. In this situation we are confronted with the impossibility of computing water volumes and application rates. After two applications the snails were reduced to 99+%, except in the lateral swampy areas which are under attack. Repopulation trends will be observed.

b. Rod formulations of NaPCP with slow dissolution rates (about 72 hours) will be field-tested. Also a preliminary lab evaluation is being carried on relative to time-concentration relationships of NaPCP with prolonged, low concentrations; and in this relationship the behavioristic reactions in response to the chemical are being studied.

c. An attempt will be made to disperse an oil concentrate of Dinex over water surfaces in swamp habitats.

Publications:

1. A Standardized "Field-Screening" Test for Molluscicides Against Aquatic Snails in Flowing Water. Luis A. Berrios-Duran, Lawrence S. Ritchie, and Henry B. Wessel. J. Parasitol. 51 (No. 2, Sect. 2): 31, 1965.

2. Molluscicidal Qualities of Three Organo-tin Compounds Revealed 6-hour and 24-hour Exposures Against Representative Stages and Sizes of Australorbis glabratus. Lyman P. Frick, and Wilma Q. de Jimenez. Bull. Wld. Hlth. Org. 31: 429-431, 1964.

3. Molluscicidal Time-Concentration Relationships of Organo-tin Compounds. Lawrence S. Ritchie, Luis A. Berrios-Duran, Lyman P. Frick, and Irving Fox. Bull. Wld. Hlth. Org. 31: 147-149, 1964.

4. Comprehensive Evaluations of Candidate Molluscicides. Lawrence S. Ritchie. Proceedings, 1st International Congress of Parasitology, Rome. In press (abstract of paper presented).

5. Molluscicidal Activity of Baited WL-8008 ("Shell" Research Ltd.) Against Australorbis glabratus. Frank J. Etges, and Lawrence S. Ritchie. Bull. Wld. Hlth. Org. (In press).

6. Molluscicidal Time-Concentration Relationships: Dinitrophenol Compounds. Lawrence S. Ritchie, and Henry B. Wessel. Bull. Wld. Hlth. Org. (In press).

7. Observations on the Effect of Schistosoma mansoni Infection Upon Fecundity of Australorbis glabratus. Frank J. Etges, and William Gresso. J. Parasitol. (In press).

8. Chlorine and Sodium Chloride Induced Resistance in Australorbis glabratus. Irving Fox, Gisela A. Rivera, Lawrence S. Ritchie, and Lyman P. Frick. J. Parasitol. (In press).

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10. The Molluscicidal Activity of Hexachlorodimethyl Sulfone (Definitive Screening). Lawrence S. Ritchie, Ida Joseph, and Wilma Q. de Jimenez. WHO Mol/Inf/18, 1964 (Special Report).

11. Definitive Screening for 14 Molluscicidal Compounds. Lawrence S. Ritchie, and Wilma Q. de Jimenez. WHO Mol/Inf/18, 1964 (Special Report).

12. Molluscicidal Activity of Organo-lead Compounds: Definitive Laboratory Screening. Lawrence S. Ritchie, Wilma Q. de Jimenez, and Ida Joseph. WHO Mol/Inf/18, 1964 (Special Report).

13. Comparative Piscicidal Activity of "Stabilized Chevreul Salt" (SCS), A Candidate Molluscicide, and Bayluscide. Luis A. Berrios-Duran, Lawrence S. Ritchie, Lyman P. Frick, and Irving Fox. WHO Mol/Inf/18, 1964 (Special Report).

14. Molluscicidal Qualities of WL-8008 Revealed by 6-hour Exposures Against Representative Stages and Sizes of Australorbis glabratus. Lawrence S. Ritchie, Lyman P. Frick, Luis A. Berrios-Duran, and Irving Fox. WHO Mol/Inf/18, 1964 (Special Report).

15. Molluscicidal Time-Concentration Relationships: WL-8008. Lawrence S. Ritchie, Irving Fox, Luis A. Berrios-Duran, and Lyman P. Frick. WHO Mol/Inf/18, 1964 (Special Report).

16. Procedures and Problems on the Evaluation and Development of Molluscicides. Lawrence S. Ritchie. WHO Mol/Inf/18, 1964 (Special Report).

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6402	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT	6. SECURITY WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
1 07 65	A. New	U	U	"NA"	NL	A. Work Unit

10a. CURRENT NUMBER/CODE: 61145011 3A014501A71Q 01 155

10b. PRIOR NUMBER CODE: 61145011 3A014501A71Q 01 06

11. TITLE: (U) Diarrheal Diseases (PR)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 Microbiology	08 61	"NA"	Other | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C	"NA"	PRIOR FY 65	2	110
		CURRENT FY 66	2	111

19. GOV'T LAB/INSTALLATION/ACTIVITY

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
"NA"	"NA"

23. KEYWORDS: Gastro-enteropathies, Microbiology, Escherichia coli, Echo viruses

24. (U) Tech Objective - The principal and subordinate technical objective is related to the importance of acute gastro-enteropathies and their potentially explosive effect on military operations as well as public health which makes study of diarrheal diseases of profound significance. Because this laboratory is located in an area where such diseases are common we are able to study them extensively from an etiologic, epidemiological and physiologic viewpoint.

25. (U) Approach - Conduct basic and clinical research in microbiology, especially in the etiological, clinical, metabolic and epidemiological aspects of diarrheal diseases. These studies are conducted primarily in children.

(U) Progress - A longitudinal study has been conducted with 26 families for one year. The most common enteropathogens producing diarrhea in these families are enteropathogenic Escherichia coli and Echo viruses type 3, 6, 7, 8, 11, 14, 15 and 17. The data revealed that the majority of the diarrhea in this group of children is of viral
26. etiology.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
"NA"	"NA"

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A014501A71Q: Communicable Diseases and Immunology

Task No. 3A014501A71Q 01: Communicable Diseases

Work Unit No. A71Q 01 06: Diarrheal Diseases (PR)

Reporting Installation: U. S. Army Tropical Research Medical Laboratory
APO New York 09851

Division: Microbiology

Period Covered by Report: 1 July 1964 - 30 June 1965

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Report Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

BODY OF REPORT

Project No. 3A014501A71Q: Communicable Diseases and Immunology

Task No. 3A014501A71Q 01: Communicable Diseases

Work Unit No. A71Q 01 06: Diarrheal Diseases (PR)

Study 1. Studies on the epidemiology of diarrheal diseases.

Progress:

The longitudinal epidemiologic study on diarrheal diseases that was started in late 1963 was gradually enlarged to include a total of twenty-five families. These are all from the same neighborhood in Cantera. They belong to the lower socio-economic strata and only a few of them have incomes of middle class range. The living conditions are for the most part quite undesirable from a sanitary standpoint, although a few families live in adequately clean and comfortable environments. See Annual Progress Report, 1964.

All family members have a medical record. Throat and stool cultures are done initially and trimonthly. The feces are examined for ova and parasites. In addition, close surveillance of the families is carried out for the detection of diarrhea cases. This is done by means of bi-weekly or more frequent visits to each house. One or more stool samples are obtained from each diarrhea case for bacterial and viral studies. The patient is examined by the physician in charge of the study whenever indicated, who gives the necessary medications and follow-up care. Acute and convalescent sera are obtained as required.

It is intended to follow every family for a period of at least one year. To date, studies on fourteen families have been completed or are near completion. Those on the remaining families will be completed by the end of 1965.

The data available on eleven of the families that were followed for at least one year reveal an average incidence of 1.18 diarrheal episodes per family per month. Families with two or more children under 3 years showed a much greater frequency of diarrheal disorders than those with older children. Out of a total of 175 diarrheal episodes that occurred in these eleven families over the period studied, 112, or 64%, occurred in the less than 3 year old group. One or several stool samples for microbiologic analysis were obtained from affected patients in only 142 diarrheal episodes. There were bacterial or viral isolations with probable etiologic significance in 65 of the 142 episodes studied. Further proof in all cases will have to await serologic confirmation which is being done.

Among the diarrheal episodes with negative bacterial or viral findings, eight in young children were associated with significant parenteral infections, and six were associated with heavy trichuriasis or Giardia infestations. At least three other episodes probably resulted from indigestion. In a few instances the patient had received small amounts of an antibiotic prior to sampling, and a few stool specimens were obtained when the corresponding diarrheal episode was just over. The latter facts may have affected adversely the number of positive isolations obtained.

Bacteria alone were recovered in 16 of the 65 episodes with positive bacterial or viral findings, thus accounting for 24.6% of the etiologic agents encountered. The group included seven enteropathogenic E. coli (6 in infants, 1 in an adult), five Alkalescens-Dispar 02, three Salmonella newport, one Shigella flexneri, and one Shigella sonnei. In one case Salmonella newport was isolated together with EPEC 0125:B15.

Viral agents alone were recovered during 49 of the 65 episodes, thus being the sole implicated etiologic agent in 75.4% of the cases with positive findings. Sixteen of these viruses have not been identified as yet. The remainder are shown in Table I. These viral episodes affected infants or small children almost exclusively. Two infected adults had very mild symptomatology. No striking seasonal variation in the incidence of diarrhea has been apparent so far, but there is a tendency for some viruses to be isolated only in the winter months and other only in the summer months, Table I.

Combined viral and bacterial agents were isolated simultaneously or from different stool samples during 13 of the 65 episodes (20%). Seven of the viruses have not been identified; Echo 8 was recovered three times, and Echo 3, Echo 20 and Polio III, respectively, were isolated once. The bacterial agents recovered from these mixed infections were enteropathogenic E. coli (predominantly 0111 and 0127), a Shigella flexneri, a Salmonella aberdeen, a Salmonella newport, and Alkalescens-Dispar 02. These combined infections were found exclusively in the infant group, except for one which was present in a 2-year old child.

There was no characteristic clinical pattern to either bacterial or viral infections. In general, the latter tended to have a shorter course, though in some infants symptoms lasted up to ten days. Fever was more frequent in the infant group than in the older children with either type of infection. Melena was reported in a patient with Salmonella newport, in a child with Shigella flexneri 3, and in an infant with an Echo 3 infection. The character of the stools was very variable otherwise, as well as their frequency. Combined infections tended to run a more protracted course than single ones.

In spite of the relative proximity of many of the families studied, diarrheal outbreaks were limited to only one household, with the exception of the Alkalescens-Dispar 02 outbreaks, described elsewhere, which affected members of two next-door families. One of these two families also had an outbreak of Salmonella newport, that caused a moderately severe

gastroenteritis in triplet siblings, and was asymptomatic in a 12 year old aunt. In addition enteropathogenic E. coli O111:B4 and O127:B8 respectively, were isolated simultaneously in at least two of the triplets on two occasions.

The remaining outbreaks observed in the eleven families under consideration were of viral etiology. The most conspicuous one occurred in the same family mentioned above, was due to an Echo 8, and was symptomatic in all five siblings. There were at least seven other instances of simultaneous isolation of the same virus in two or more family members with diarrhea. The patients affected during these outbreaks were usually infants or young children.

In summary, viral agents were recovered with far greater frequency (at least three-fold) than enteropathogenic bacteria from diarrhea patients of eleven families studied for at least one year. There was no characteristic clinical picture to either type of infection. Both types of agents affected infants and young children much more frequently than other age groups. Diarrheal outbreaks were usually limited to one household and were of viral etiology in the majority of instances. Significant parasitic infestations causing diarrhea were found in only two children of the reported group.

Summary:

The longitudinal epidemiologic study on diarrheal diseases that was started in September 1963 was gradually enlarged to include a total of twenty-five families. These families are all from the same neighborhood in Cantera. They belong to the lower socio-economic strata and only a few of them have incomes of middle class range. The living conditions are for the most part quite undesirable from a sanitary standpoint, although a few of the families live in adequately clean and comfortable environment.

The data available on eleven of the families that were followed for at least one year revealed an average incidence of 1.18 diarrheal episodes per family per month. Families with two or more children under 3 years showed a much greater frequency of diarrheal disorders than those with older children. Of a total of 175 diarrheal episodes that occurred in those eleven families over the study period, 112 or 64%, occurred in the less than 3 year old group. Of the diarrheal episodes with positive microbiological findings 24.6% were due to bacteria and 75.4% to enteric viruses. There were mixed viral and bacterial infections in 20% of the cases. There was no characteristic clinical picture to either type of infection. Significant parasitic infestations causing diarrhea were found in only two children of the reported group.

Study 2. Cross-sectional serum survey for viral antibodies.

Progress:

A serological survey of neutralizing antibodies against given enteroviruses isolated from Puerto Rican children is being conducted in an attempt to ascertain the incidence of enteroviral infections in this island. A total of 1,000 sera are under study. These were obtained from the urban population of the San Juan area (Barrio Obrero, Puerta de Tierra), the rural population of Guaynabo, a town 11 miles southeast of San Juan, and two isolated communities, Manzanillas in the southern part of the island and Cialitos in the central mountainuous region of Puerto Rico. The population in general was of low socio-economic status, living in crowded and unsanitary conditions. In many instances, serum specimens were obtained from all members of given families. The viruses selected for this study were Echo type 3, 7, 8, 9, 11 and Coxsackie A9. Serum dilutions of 1:10 in Hank's Balanced Salt solution were employed. The neutralization tests were carried out by mixing 0.2 ml of the serum dilution with 0.2 ml of a dilution containing 200 $TCID_{50}$ virus per 0.1 ml. The mixture was incubated at 25°C for one hour. A 0.1 ml aliquot of the serum-virus mixture was inoculated into each of 2 tubes of monkey kidney monolayers. The presence or absence of antibody in each given serum was determined by the inhibition or lack of inhibition of the cytopathogenic effect of the virus.

To date sera have been tested for the presence of antibodies for ECHO type 3, 7, 8, 9 and Coxsackie A9. The results shown in Table 2 demonstrate that the five viruses studied are prevalent in those areas. These viruses prevail more in urban or crowded areas than in isolated areas as shown in Table 3. Cialitos, which is an isolated community, has the lowest percentage of persons with neutralizing antibodies for the viruses surveyed.

Exposure to the viruses studied occurred early in life. The percentage of persons possessing neutralizing antibodies increases rapidly and reaches a maximum at nine years of age, Table 3. It appears that these viruses stimulate neutralizing antibodies that after a certain age begin to decline in titer. This is certainly true for ECHO 7 virus, Table 4. The other viruses also have this tendency but more data is necessary to reach a definite conclusion.

Summary:

A serological survey of neutralizing antibodies against some of the enteric viruses was conducted in an attempt to ascertain the incidence of certain enteroviral infections in different areas of Puerto Rico. The viruses employed in the survey were: Echo type 3, 7, 8, 9, and Coxsackie A9. One thousand sera from five different areas of Puerto Rico were used in the study. Exposure to the five viruses studied occurred early in life. The percentage of persons possessing neutralizing antibodies increases rapidly reaching a maximum around the age of nine years in all the viruses

The patterns of occurrence of antibody showed that the five viruses spread readily within households.

Study 3. Studies on the passive immunity of the infant.

Progress:

Preliminary studies showed that the immune protein content of human colostrum was exceptionally high and that these proteins behave as antibodies in the different immunological systems employed. The existence of these antibody-like substances in maternal tissue, the diversity of ways in which they react in vitro against several antigens, and the relative concentrations in which they appear in the various tissues prompted us to study their effect on the immune status of the newborn.

Placental tissues were collected from 36 childbirths with the cooperation of the San Juan City Hospital Obstetrics Department staff. Placental extracts, maternal blood serum, cord blood serum, and colostrum samples up to the tenth day post-partum were assayed by various serological methods for the quantity and quality of their immune protein contents.

Hemagglutinating antibodies for E. coli antigens O111:B4, O55:B5, O126:B16, and O119:B14, were assayed in all samples. In two sets of samples agglutinating titers for S. typhi somatic and flagellar antigens were measured.

No antibodies for these antigens were detected in the placental extracts. With rare exception of low or no titers, the mother blood sera showed hemagglutinating titers of moderate order towards the E. coli antigens with the following averages:

H. A. ANTIGEN	MATERNAL SERA
E. coli O111:B4	1:36
" " O55:B5	1:48
" " O126:B16	1:48
" " O119:B14	1:52

Approximately one fifth of the cord blood sera showed hemagglutinating titers against any one of the four E. coli antigens. These titers were of low order in general (about one fourth of the maternal serum titers) and only appeared in those samples in which the maternal blood had a high titer for the particular antigen.

All colostrum samples showed titers against the four E. coli antigens. In general, these titers reached magnitudes sixteen times higher than the

maternal serum titers. A tendency was evident for the colostral titers to decrease as the time interval post-partum increased. In only 4 of the 56 titers compared was there a direct increase in titer with time post-partum of the colostrum. The average titers of the 34 samples representing 21 parturitions were as follows:

H. A. ANTIGEN	COLOSTRUM	TITER RATIO COLOST/MAT SERUM
E. coli 0111:B4	1:984	27.3
" " 055:B5	1:588	12.3
" " 0126:B16	1:540	11.3
" " 0119:B14	1:684	13.1

These results suggest that antibodies against the enteropathogenic bacteria tested did not pass through the placenta. This was shown by the absence of antibodies in the majority of the cord blood serums tested. On the other hand, antibodies against the same antigens were concentrated in the colostrum. These results suggest that passive immunity against enterobacteria could be transferred from mother to newborn via colostrum. Studies done with some enteric viruses and influenza virus showed that the cord blood serum had the same level of antibodies as the mother blood serum and that neutralizing antibodies were not concentrated in the colostrum. The passive immunity in the case of the enteric viruses tested and influenza virus in the newborns is probably acquired via placenta or by both via colostrum and via placenta.

Absorption experiments done with antibodies present in the colostrum showed that they were specific for a given antigen. Single step absorptions with whole cell formalized antigens were carried out. The results are shown in Table 5. Heterologous absorption only occurred in one case and was with mother blood serum.

Total protein determinations were done on all samples and the protein fractions of each were grossly differentiated by electrophoresis at 2.5 milliamperes for 16 hours. Placental extracts showed very low protein content with a short migration range. This was probably due to excessive dilution of the tissues (1:5) which may also account for the failure to detect measurable antibodies in these titers. Concentration of these extracts by lyophilization will be attempted before serological and chemical experiments are repeated on them.

Colostral proteins ranged from 1.3 to 11.16 grams percent with a mean of 3.71 grams percent. On macroelectrophoresis, these proteins could not be finely differentiated, but at least three peaks were evident in the gamma globulin area. No albumin could be detected by electrophoresis.

Mother blood serum proteins showed an average of 5.86 with values as low as 4.7 and as high as 6.99 grams percent.

The average total proteins of the cord blood sera was 5.83 with low value of 4.71 and high value of 7.99 grams percent.

Summary:

Studies done in this laboratory have shown that the immune protein content of human colostrum is exceptionally high. These proteins behave as antibodies in the different immunological system employed. Placental extracts, maternal blood serum, cord blood serum and colostrum samples were assayed by various serological methods for the quantity and quality of their immune protein contents. Hemagglutinating antibodies for Escherichia coli antigens O111:B4, 055:B5, O126:B6 and O111:B14 were assayed in all samples. No antibodies for these antigens were detected in the placental extracts. The mother blood sera showed hemagglutinating titers of moderate order against these antigens (1:48). The cord blood sera showed titers of very low order (about one fourth of the maternal serum titers). All colostrum samples showed titers against the four E. coli antigens. In general, these titers reached magnitudes sixteen times higher than the maternal serum titers. Similar results were obtained with influenza and some enteric viruses studied with the difference that antibodies were not concentrated in the colostrum.

Study 4. Studies with arboviruses.

Progress:

In a general investigation designed to study the immunological significance of cross-protection in the arboviruses, it has been decided to study the relationship between the immature forms and cross-protection in members of the equine encephalitis viruses. The outline for this work was given in last year progress report. The reports in the literature are contradictory in regard to the presence of group A arboviruses in this island. A serum survey has been started to answer this question. In addition this program will be expanded to include group B arboviruses, especially some basic studies on the biology of Dengue virus.

Summary:

These investigations have been initiated with a serum survey to determine which are the arboviruses prevailing in this island.

Study 5. Diagnostic bacteriology.

Progress:

A total of 1,652 specimens were received by the Department of Bacteriology as follows:

1. Fecal Samples: 1,022 samples were processed by cultural methods for enteric pathogens such as Salmonella, Shigella, enteropathogenic E. coli and coagulase positive staphylococci. In addition, the detection of enteropathogenic E. coli was done by fluorescent antibody techniques. The identification of pathogens was carried out to completion by both serological and biochemical methods except in the case of Salmonella isolates that were sent to Dr. W. H. Ewing at CDC for serological typing after being grouped in this laboratory. The following organisms were isolated and identified during the period:

	Isolates
A.	
Salmonella sp.	15
newport	9
senftenberg	2
infantis	1
oranienburg	1
enteriditis	1
anager	1
B.	
Shigella sp.	14
flexneri 3	9
flexneri 2	2
sonnei 1	2
sonnei 2	1
C.	
Alkalescens-Dispar group	26

D.

Enteropathogenic E. coli	80
0111:B4	18
055:B5	15
0125:B15	14
0126:B16	10
0124:B17	6
0127:B8	6
026:B6	4
0128:B12	4
086:B7	2
0119:B14	1

Employing commercial conjugated antisera, approximately 4,500 smears were stained and observed for fluorescence. The number of detections by fluorescence was much larger than the number of cultural isolations as in previous experience; 34% of the samples were positive by fluorescence and 7.8% by both cultural and fluorescence.

2. Throat Cultures: 538 specimens were received and cultured for upper respiratory tract pathogens.

a. Beta-hemolytic streptococci isolated:	134
b. Coagulase positive staphylococci isolated:	144
c. Monilia isolated:	5
d. Klebsiella isolated:	3

3. Miscellaneous Samples: 92 miscellaneous samples such as urine, eye cultures, ear cultures and laboratory animals pathological tissues were received for culture or sterility tests.

Summary:

One thousand and twenty-two stool samples were processed by cultural methods for enteric pathogens. The percentage of isolation of enteric bacteria was as follows: Salmonellae, 1.5%; Shigellae, 1.4%; enteropathogenic E. coli, 7.8%; Alkalescens-Dispar group, 2.5%.

Five hundred and thirty-eight throat specimens were cultured and the following bacterial agents were isolated: coagulase positive staphylococci, 27%; hemolytic streptococci, 25%; Monilia, 0.9%; and, Klebsiella, 0.6%.

Study 6. An outbreak of gastroenteritis caused by a member of the Alkalescens-Dispar group.

Progress:

The Alkalescens-Dispar group of organisms is probably the most obscurely defined in the Enterobacteriaceae family. The diversity of opinion in regard to nomenclature, taxonomy, biochemical activity and antigenic structure of members of this group has existed since the first decade of the century. In 1943, Stuart et al. (1) isolated a series of antigenically identical or closely related group of organisms ranging from Shigella alkalescens to Escherichia coli from an epidemic of gastroenteritis in a nursery. The subsequent works of Carpenter, Stuart, and Ewing led the Enterobacteriaceae Subcommittee of the International Association of Microbiologists in its 1950 meeting (2) to remove the Alkalescens-Dispar group from the genus Shigella. Because of the biochemical and antigenical similarities between this and the Escherichia groups the Subcommittee hinted that the Alkalescens-Dispar group should be considered as members of the E. coli group. On the other hand Berge's Manual of Determinative Bacteriology in its 1957 edition includes S. dispar and S. alkalescens in the Shigella genus. From the medical point of view these considerations would merely bear their due academic interest. However, in the extant confusion, the group as a whole has lost its pathogenical prestige to the extent that individual strains, when isolated from cases of acute gastroenteritis, are not even considered as possible etiological agents.

Studies to determine the etiological role of an Alkalescens-Dispar strain are being conducted as a satellite of the Cantera Epidemiological Project. This microorganism was isolated from all but one of the 8 members of one of the families and from 2 children from a next door 4 member family. These two families lived in close association and shared at times a 12 year old baby sitter who is a member of the larger family.

The clinical picture consisted of diarrhea characterized by several bowel movements per day, abdominal pain, and vomiting. In three of the children the diarrhea bout was accompanied by low grade fever. The duration of symptoms ranged from 2 to 16 days, except for the 12 year old baby sitter who exhibited no associated symptomatology throughout the period.

The microorganism isolated was a non-motile Gram negative short rod which attacked the following substrates with the production of acid but no gas: arabinose, dextrose, maltose, mannitol, rhamnose and sorbitol. It failed to acidify media containing adonitol, dulcitol, inositol, lactose, salicin or sucrose. It did not hydrolyze urea, and was indol and methyl red positive, Voges-Proskauer and citrate negative. In vitro, it was susceptible

to furoxone, chloromycetin and colymycin, moderately susceptible to kanamycin and neomycin and resistant to novobiocin, tetracycline, erythromycin, penicillin and streptomycin. A strong antigenic relation with E. coli 0124:B17 was detected by agglutination and fluorescence. All the isolates agglutinated strongly in polyvant Alkalescens-Dispar antiserum, but failed to agglutinate in monovalent sera prepared at WRAIR. One of the cultures was sent to Dr. W. H. Ewing at CDC who identified it as Alkalescens-Dispar 02.

Humoral response to the infection was demonstrated by rise in hemagglutinating titers in each of the cases as is shown in Table 6.

Summary:

A member of the Alkalescens-Dispar group produced an outbreak of diarrhea in two neighbor families that have been under study for more than a year. These families are two of those under study in the Cantera Epidemiological Project. There is evidence that the etiologic agent of this outbreak was Alkalescens-Dispar 02.

Study 7. Complement fixation test for adenoviruses.

Progress:

Although the adenoviruses were originally discovered in children, the demonstration of their importance in the respiratory disease problem in military recruits tended to divert attention from their importance in pediatrics. It is the feeling of many workers that the greatest significance of the adenoviruses to public health lies in the pediatrics area. These viruses are among the most frequent and earliest infecting viruses of the young infant; although severe disease is relatively infrequent, the large numbers of infections that occur make their contribution to illness in young children quite considerable.

A preliminary study was done to determine the incidence of infections with adenoviruses in the families under study in the Cantera Epidemiological Project. One hundred and fifty-five patients were surveyed for a year. At least five different sera were done from each patient. Of the 155 original patients 101 had complement-fixing antibodies at the beginning of the study and twenty-one became positive subsequently. Of these, 16 had a common cold with or without fever, two asthma, one earache and in the other two no information was available for the period during which the experience with the virus probably took place. The remainder of the patients were negative throughout the year. There was no drop in titer of the complement-fixing antibodies in a year period in the patients that were positive at the beginning of the study. It was found that children at birth had complement-fixing antibodies for adenoviruses but these antibodies disappeared after the first month of life.

Summary:

Six hundred and six sera from the Cantera Epidemiological Project were tested for the presence of complement-fixing antibodies for adenoviruses. One hundred and fifty-five patients were surveyed for a year; of these, one hundred and one were positive at the beginning of the study. Twenty one became positive subsequently. Of these, 16 had common cold, two asthma, one earache and for two no information was obtained.

Study 8. Diagnostic virology.

Progress:

A total of 1,375 specimens were received for the isolation of enteric viruses in monkey kidney cells. The following isolations were obtained:

Specimens	Total number	Viral isolation		Percent
Rectal swabs	840	Echo	80	9.5
		Coxsackie	13	1.6
		Polio	16	1.9
		Not identified	40	4.8
			149	17.8
Throat swabs	535	Echo	5	0.9
		Not identified	3	0.6
		Unknown	1	0.2
			9	1.7

Summary:

One thousand three hundred and twenty-five specimens were processed for the isolation of enteric viruses in monkey kidney cells. The percentage of viral isolation was 10% in routine samples. In outbreaks the recovery of viral isolation was of 75-100%.

Study 9. A study of an epidemic diarrheal outbreak in Cidra.

Progress:

Diarrheal disease of infants has attracted attention for many years. Several viruses were isolated by inoculation of experimental animals, but their significance is uncertain and in some cases the agents may have originated from the experimental animals rather than from the material

inoculated. Application of tissue culture techniques led to the isolation of numerous agents, most of which are now classified as enteroviruses, from apparently healthy children and from those suffering from poliomyelitis-like, diarrheal, and other diseases. Since enteric virus infections and diarrhea are both common events in childhood, proof and case relationship has been difficult to obtain. Comparisons of the frequency of virus isolations from diarrheal and control non-diarrheal children have shown association between diarrhea and infection with certain Echo viruses.

It seems that diarrhea can be caused by infections with a variety of viruses, some cultivable and some, like the virus of infectious hepatitis, very fastidious to be grown in the laboratory. Fortuitous association between virus infection and diarrheal disease is especially common in children and interpretation of virological studies requires special care in this group. Enteric viruses apparently spread in the community largely through young children, the particular type, dominant at any given time being influenced by the general level of immunity of this group. Thus the predominant enteric viruses vary from time to time and from place to place. Several different viruses may be prevalent in a community at the same time or in succession. An outbreak of non-viral diarrhea may coincide with an episode of prevalence of a particular enteric virus. An investigation of such an outbreak was conducted by this laboratory in the town of Cidra this year.

During the second week of January an increase of diarrhea patients was noted in the Health Center of Cidra. At the beginning of February a group of workers from the Tropical Research Medical Laboratory went to the Cidra Health Center to take samples for bacteriological and virological studies. Acute blood was taken from every patient. A convalescence blood was taken 21 days later from patients that willingly cooperated with the study.

The media used in the bacteriological studies was MacConkey, SS and Selenite. Monkey Kidney cells (Rhesus) were used for the virological studies.

The enteropathogens isolated in Cidra are shown in Table 7. The high recovery of enteric viruses (49%) immediately suggested that we were dealing with an outbreak of virological origin. Table 8 shows the enteric viruses isolated. Echo types 11 and 7 viruses were the most frequently isolated with 47% and 30% respectively.

Echo 11 virus was found to be spread throughout the municipality as shown in Table 9. E. coli and Shigella did not seem to play a primary role in this outbreak.

The studies done in the hospitalized patients in the Cidra Health Center showed that the enteric viruses were again present in high number, (63% of the patients) but in this case Echo 7 was more frequently found than Echo 11, Table 10. This strongly indicates that Echo 7 virus was

present as a hospital outbreak and that Echo 11 virus was present in the community.

The results of the serological studies are shown in Table 11. Of the paired sera collected 85% showed an increase in neutralizing antibody titer for Echo 7 and 100% showed an increase in neutralizing titer to Echo 11 virus, therefore, 85% of the patients had a mixed infection during the period. These sera were also tested for hemagglutination inhibition antibody titers and showed the same results obtained for the neutralizing antibodies.

During the visit to Cidra, it was noticed that the mothers were giving from 1 to 20 days as the onset of the diarrhea. It was suspected at that time that we were dealing with more than one etiologic agent. The duration of diarrhea in patients that showed rise in titer to Echo 7 and Echo 11 viruses is shown in Table 12. Some of the children had also a bacterial infection. These results clearly explain the long diarrheal periods suffered by these children. It seems that they were suffering one infection after the other. It would be difficult to determine if dual infections occurred simultaneously. Perhaps the cases in which a short period of diarrhea and a rise in antibodies was shown for both viruses probably represent a dual infection.

At the same time of the Cidra outbreak several cases were reported in Bayamon and Rio Piedras. Some studies were conducted in these two areas, and Echo 7 and Echo 11 viruses were found to be responsible for the outbreak.

Earlier in the winter (December) an outbreak was reported in Ponce. Echo type 8 virus was the one responsible for this outbreak. Ponce is in the southern part of the island.

Summary:

An outbreak of diarrhea among children was studied in the town of Cidra. There were around 500 cases. Two children died as a consequence of the outbreak. Echo 11 and 7 viruses were found to be responsible for this outbreak. The Echo 11 virus was spread throughout the town; while Echo 7 virus was found among the hospitalized patients. Some of the children had long periods of diarrhea (10-30 days). The children entered the hospital with Echo 11 virus infection, once in the hospital they acquired Echo 7 virus, and in some cases these viral infections were preceded or followed by an enteropathogenic E. coli infection that also was found to be in the hospital. This accounted for the long period of diarrhea in some of the children.

Study 10 Nonspecific reactions in the diagnosis of enteropathogenic E. coli by immunofluorescence.

Progress:

While employing fluorescent antibody (FA) techniques for the past three years in the detection of enteropathogenic E. coli (EPEC) ample data have been accumulated. It has been found that several nonspecific reactions can occur that render these methods misleading and lacking in the degree of reliability desired in a routine laboratory test.

In order to compare our findings with those of Cherry and co-workers (3), a group of patients from the San Juan City Hospital was selected as most appropriate since this hospital was one of the sources of patients studied by them in 1961. The following is a comparison of the data of Cherry et al. with those obtained in this laboratory.

	CHERRY et al.	TRML
Number of Samples	291 (100%)	70 (100%)
Culture positive	72 (24.7%)	26 (37.1%)
FA positive	106 (36.4%)	43 (61.4%)
Culture/FA	0.68	0.60

In most of our patients, rectal swabs or fecal samples were obtained and tested for at least three consecutive days. This circumstance probably accounts for our higher percentage of isolations. In spite of the high percent of positive cultures, the culture positive to FA positive ratio was lower than that obtained by Cherry and his group.

Three possible sources of error were studied: (1) Basic technique (2) Quality of reagents (3) Interpretation of observations. No faults in the basic technique were apparent and this possibility was tentatively eliminated. Since at the end of the period the technician engaged in the FA work had acquired nearly a year of experience and the reagents then available were from the same source as those used initially, an attempt to appraise the two remaining possibilities was made. The saline fecal suspensions of those samples previously scored FA positive culture negative, which had been stored at -40°C were allowed to stabilize to room temperature for two hours. Two smears were made from each suspension. One was stained with the same fluorescent globulin that had shown a positive reaction in the original test and the other stained with conjugated normal rabbit serum. Several attempts were made to culture the suspensions in order to make a more thorough search to confirm the FA results, but this practice was discontinued when the cultures yielded very few or no colonies for serotyping by agglutination.

The effect of this re-evaluation on the previous data is shown below.

	CHERRY et al.	TRML ORIGINAL	TRML REEVALUATED
Culture Positive	24.7%	37.1%	—
FA Positive	36.4%	61.4%	54.3%
Culture/FA	0.68	0.60	0.68

Of the 149 samples re-evaluated, 93 were confirmed as FA positive, the remaining 56 rated as "false positives". Some of the microscopic findings leading to erroneous identifications were: artifacts, inconsistent cellular morphology, no fluorescent organisms seen, and faintly staining organisms.

The appearance of artifacts was detected by subjecting the microscopic field to white light. Under this kind of illumination the observed structures had no semblance to an organized bacterial cell and were present in both the specific immune globulin and the normal rabbit serum. Inconsistent cellular morphology as a contributory factor in the finding of false positives was attributed to coccoid cells stained with equal intensity by both the immune and the normal globulin. This and the other two factors were probably due to deficient specificity of the conjugates used.

The frequency of false positives among the different E. coli serotypes is shown below:

0124:B17	20	0111:B4	4
0125:B15	11	086:B7	4
0128:B12	7	0119:B14	2
0126:B16	6	055:B5	2

The predominance of false positives in the case of E. coli 0124:B17 is striking and was due to nonspecific staining in 14 of the 20 cases.

It is evident that technical skill and experience in the interpretation of observation are strict requirements for the application of FA methods as a routine laboratory test. Furthermore, it has been demonstrated that the inadequacy of commercial reagents is a risk to the proper identification of E. coli by FA methods.

However, there were still 32% of the detections by FA that could not be confirmed by standard cultural and serological methods. It was noticed

that failure to isolate the detected serotype resulted mostly from the samples in which the ratio of fluorescent organisms to the commensal bacteria was less than 25%. It must be realized that viable and unviable cells are equally stained by fluorescent antisera. This may account, to some extent, for the discrepancy of the findings by the two methods, depending on such factors as antibiotic therapy, host-parasite status, and manipulation and age of the samples.

Davies and Ewing (4) have pointed out the existence of intra and extra species share of antigens of the E. coli group. It is probable that many other enteric bacteria have escaped the investigations of these authors.

Studies are being conducted at this laboratory to determine to what extent other organisms "cross react" with the commercial sera that are presently available to diagnostic laboratories. So far it has been demonstrated that at least two Klebsiella biotypes "cross react" with certain batches of conjugated rabbit antisera. The probability of this "cross reactions" of being specific reactions due to true anti-Klebsiella antibodies in the so-called normal rabbit has been demonstrated by cross absorption of the agglutinating and fluorescent antisera. These heterologous reactions have been detected in sera prepared against E. coli 026:B6, 055:B5 and 0124:B17. In addition several isolates of Alkalescens-Dispar 02 were found to react both by agglutination and by fluorescence in E. coli 0124:B17 antisera.

By the investigations so far completed, the opinion of many workers in this field has been confirmed in that FA methods alone, as they stand now, cannot be deemed efficient laboratory tools, except when used as screening and confirmatory devices.

Summary:

The fluorescent antibody technique has been evaluated for the past three years in this laboratory for the detection of enteropathogenic E. coli. It has been found that several nonspecific reactions occurred when commercial reagents were used. The method is misleading and does not have the degree of reliability desired in a routine laboratory test.

Publications:

1. An outbreak of diarrhea at the San Juan City Hospital Department of Pediatrics. Aida L. Guardiola-Rotger, and Victor A. Lopez. The Journal of Pediatrics, Vol. 65 (1): 81-91, 1964.

2. Endemiological Studies on Diarrheal Diseases in Puerto Rico. Aida L. Guardiola-Rotger, Aurea Munoz, Victor A. Lopez, and Diana E. Gadea. (Accepted for publication by the American Journal of Diseases of Children).

3. A Study of an Epidemic Diarrheal Outbreak in Puerto Rico. Julio I. Colon. (Presented at the Annual Meeting of American Academy of Pediatrics, San Juan, Puerto Rico, April 30, 1965).

4. Studies in the Passive Immunity of the Infant. Victor A. Lopez, and Julio I. Colon. (In preparation).

5. An Outbreak of Gastroenteritis Caused by a Number of the Alkalescens-Dispar group. Victor A. Lopez, Aurea Munoz, and Julio I. Colon. (In preparation).

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 6403	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
1 07 65	A. New	U / U	"NA"	NL	A. Work Unit

10a. CURRENT NUMBER/CODE: 61145011 3A014501A71Q 01 156

10b. PRIOR NUMBER CODE: 61145011 3A014501A71Q 01 07

11. TITLE: (u) Parasitic Diseases (PR)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 Microbiology	01 64	"NA"	Other | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST	a. PROFESSIONAL MAN YEARS	b. FUNDS (In thousands)
C	"NA"	PRIOR FY 65	1	95
		CURRENT FY 66	1	95

19. GOV'T LAB INSTALLATION. ACTIVITY

NAME: Hqs US Army Medical Research & Dev Comd
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20. PERFORMING ORGANIZATION

NAME: U. S. Army Tropical Research Med. Lab.
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21. TECHNOLOGY UTILIZATION	22. COORDINATION
"NA"	"NA"

23. KEYWORDS: Schistosomiasis, Repellents, Cercariae

24. (U) Tech Objective - Evaluation of selected topical and systemic compounds for individual protection against schistosomiasis and therapeutic activity in another class of compounds.

25. (U) Approach - Topical compounds in undiluted form or in 20% mixture in siliconized ointment applied to tails of mice which are then exposed to cercariae after prescribed periods. Systemics injected IP daily X 10, with exposure on 5th day. Therapeutics administered IP daily X 3 in mice with 40-42 day old infections. Activity determined by oogram 1 and 7 day post-treatment. Necessity for in vivo testing with attendant holding period for animals, does not make for rapid processing of compounds.

26. (U) Progress - Thirty-two topical repellents tested; 9 gave complete protection for 6 hours; 20 gave at least 97% relative protection in same period. Eleven systemics tested preliminarily gave no protection; other compounds are under test. Of drugs tested therapeutically, 5 of 65 gave sufficient evidence of activity to merit further testing.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
"NA"	"NA"

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A014501A71Q:	Communicable Diseases and Immunology
Task No. 3A014501A71Q 01:	Communicable Diseases
Work Unit No. A71Q 01 07:	Parasitic Diseases (PR)
Reporting Installation:	U. S. Army Tropical Research Medical Laboratory APO New York 09851
Division:	Medical Zoology
Period Covered by Report:	1 July 1964 - 30 June 1965
Professional Authors:	Lyman P. Frick, Colonel, MSC Alfred Hernandez, Captain, MSC
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Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

BODY OF REPORT

Project No. 3A014501A71Q: Communicable Diseases and Immunology

Task No. 3A014501A71Q 01: Communicable Diseases

Work Unit No. A71Q 01 07: Parasitic Diseases (PR)

Study 1. Evaluation of insect repellents for prevention of schistosomiasis mansoni.

Progress:

In a continuation of work reported previously, a total of 39 topical insect repellents were evaluated for cercaricidal activity in a standardized in vitro test. For this test the repellent in undiluted form or mixed in a 20% combination with a siliconized ointment base, was lightly smeared on a watch glass and then tested against cercariae at hourly intervals for 3 hours. In each hourly period the treated watch glass is air-dried for 15 minutes and then wet with water for 15 minutes. The cercarial exposure takes 30 min., therefore, the glasses are exposed to 3 washings totaling 2¼ hours out of the 3 hours. In undiluted form, 16 compounds killed all cercariae through 3 hours and one killed at least 97% of the cercariae. The remainder of 22 did not meet either of these standards. A total of 36 compounds were selected for testing in the Covicone mixture. Of these 17 killed all cercariae through 3 hours and 5 more killed 97% of cercariae. In vivo testing is projected for those compounds giving 100% kills.

Summary:

Of 39 topical insect repellents tested in undiluted form in a standardized in vitro test, 16 killed all cercariae through 3 hours and 1 killed 97% of cercariae. Of 36 compounds blended in a 20% mixture with Covicone ointment base, 17 killed all cercariae through 3 hours and 5 more killed 97% of the cercariae.

Study 2. Preliminary screening of drugs for therapeutic activity against Schistosoma mansoni in mice.

Progress:

Through the courtesy of investigators at the National Cancer Institute, NIH, a number of drugs have been made available for evaluation of therapeutic activity against experimental schistosomiasis mansoni in mice. Preliminary screening of these drugs is in progress; for this the oogram technique of Pellegrino is being used. Mice with 6-week infections are treated with 1/8 of the maximum tolerated IP dose subcutaneously in corn oil daily for 3 days. Half the mice are necropsied on the

day following completion of treatment and the remainder 7 days later. Evidence of activity is looked for in changes in the proportion of immature eggs to mature ones in the intestinal wall. To date 122 drugs have been screened in this manner. Four of these show evidence of activity and will be tested more exhaustively. 118 additional drugs are in the process of being screened.

Summary:

Four of 122 drugs tested with the oogram technique of Pellegrino show evidence of activity and will be subjected to more exhausting testing. 118 other drugs are in the process of being screened.

Study 3. Evaluation of certain drugs as systemic cercaricides in mice.

Progress:

Two groups of rationally designed compounds have been made available for evaluation in mice. These compounds are deposited in the skin following administration where it is possible they might exert adverse effects on cercariae as they penetrate the skin. An initial group of 11 drugs were administered IP to mice daily for 6 days prior to exposure to cercariae, and then for 6 more days thereafter. None of these drugs exhibited any evidence of prophylactic activity. Another group of 65 such drugs is being evaluated at this time.

Summary:

None of 11 drugs that are deposited in the skin following administration exhibited any prophylactic activity against S. mansoni in mice. Another group of 65 such drugs are presently being evaluated.

Study 4. pH and temperature parameters for cercaricidal activity of chlorine.

Progress:

Tap water solutions of sodium hypochlorite were tested in a factorial design experiment. Results show that cercaricidal activity of these solutions was primarily related to pH and only secondarily to temperature. In solutions at pH 5.0, 7.5, and 10.0, activity was invariably highest in the first and somewhat lower in the second, neither being affected appreciably by temperature in a range of 20° to 40°C, while at pH 10.0 activity was negligible at 20° but markedly increased at 40°. Thus, with 5.0 ppm free available chlorine at 20°C, cercariae of S. mansoni were inactivated in 1-2½ minutes at pH 5.0, in 3 minutes at pH 7.5 and in 28-30 min. at pH 10.0 or in about 1/5 this time at 40°C. These times were reduced only slightly by concentrations up to 14-16 ppm. Minimal concentrations inactivating cercariae in a

30-min. contact period at 20°C were 0.3 ppm at pH 5.0, 0.6 ppm at pH 7.5 and 5.0 ppm at pH 10.0.

Summary:

A factorial design experiment involving combinations of chlorine concentration X pH X temperature shows that pH is the principal determinant of cercaricidal activity by chlorine, and further, that this activity is closely related to the concentration of hypochlorous acid formed in chlorine solutions.

Publications:

1. Influence of pH and Temperature on the Cercaricidal Activity of Chlorine. Lyman P. Frick, and George V. Hillyer. Journal of Parasitology 51 (No. 2, Sec. 2): 32, 1965.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6408	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
1 07 65	C. Termination 1 07 65	U / U	"NA"	NL	A. Work Unit

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 086

10b. PRIOR NUMBER CODE: 61130011 3A013001A91C-1

11. TITLE: (U) Study of Human Chromosomes in the Tropics (PR)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 Biology	0863	"NA"	Other | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN YEARS	b. FUNDS (In thousands)
C	"NA"	PRIOR FY 65	1	10
		CURRENT FY 66	None	None

19. GOV'T LAB INSTALLATION ACTIVITY
NAME
ADDRESS Hqs U.S. Army Medical Res & Dev Command
Washington, D. C. 20315

RESP. INDIV. Dacquisto, M. P., Lt Col
TEL Ft Brooke, P.R. 722-0000 Ext. 2101

20. PERFORMING ORGANIZATION
NAME U. S. Army Tropical Research Med Lab
ADDRESS APO New York 09851

INVESTIGATORS PRINCIPAL ASSOCIATE Dacquisto, M. P., Lt Col
Jones, L. P., Capt
TEL Ft Brooke P.R. 722-0000 Ext. 2101 TYPE

21. TECHNOLOGY UTILIZATION: "NA"

22. COORDINATION: "NA"

23. KEYWORDS: Human Chromosomes, Malabsorptive Diseases, Radiotherapy

24. (U) Tech Objective - This study of human chromosomal patterns found in malabsorptive diseases, high-level radiotherapy, congenital heart disease and newborn-infant abnormalities is to find patterns or variations suggestive of or useful as an aid for prognosis, diagnosis or treatment. Secondarily it is to determine if peripheral blood cultures are an accurate model for the individual's genetic pattern.

25. (U) Approach - Unique chromosomal patterns or aberrations, if any, will be studied in malabsorptive conditions and high-level partial body radiotherapy (these patients will be followed for several months). Various cardiac defects and several infant-neonatal abnormalities will be studied to determine if the defects are genetically derived. Various contributors will select and submit these cases. Tissue culture techniques, high resolution and accumulation of adequate numbers for experimental validity may require considerable effort.

26. (U) Progress (Aug 1963 to termination July 1965) - From August 1963 to July 1965, 680 chromosome samplings were studied. Altered or abnormal chromosome patterns could not be found with any degree of reliability in specific tropical diseases such as sprue and other malabsorption conditions. Some alterations of peripheral blood chromosomes were found in patients receiving high-level radiotherapy and this work continued separately by the Institute of Nuclear Medicine. Six cases of Klinefelter's and two of Turner's syndrome and 6 cases of mongolism were confirmed. Several neonatal and postnatal abnormalities were confirmed as congenital rather than genetic in origin.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
"NA"	"NA"

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
FY+1	

DD FORM 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A013001A91C: In-House Laboratory Independent Research

Task No. 3A013001A91C: In-House Laboratory Independent Research

Work Unit No. A91C-1: Study of Human Chromosomes in the Tropics (PR)

Reporting Installation: U. S. Army Tropical Research Medical Laboratory
APO New York 09851

Division: Director's Office
Veterinary

Period Covered by Report: 1 July 1964 - 30 June 1965

Professional Authors: M. P. Dacquisto, Lt Col, MC
L. P. Jones, Captain, VC

Report Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

BODY OF REPORT

Project No. 3A013001A91C: In-House Laboratory Independent Research

Task No. 3A013001A91C: In-House Laboratory Independent Research

Work Unit No. A91C-1: Study of Human Chromosomes in the Tropics (PR)

Progress:

Approximately 680 chromosomes samplings have been completed. Altered or abnormal chromosome patterns could not be found with any degree of reliability in specific tropical diseases such as those of malabsorption. Some alterations of the peripheral blood chromosomes were found in patients receiving very high level radiotherapy. This work unit has been terminated as a result of the principal and associate investigators rotating to CONUS.

Summary:

Altered or abnormal chromosome patterns could not be found with any degree of reliability in specific diseases such as those of malabsorption.

RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6407	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / SUM	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
1 07 65	A. New	U / U	"NA"	NL	A. Work Unit

10a CURRENT NUMBER CODE: 61130011 3A013001A91C 01 087

10b PRIOR NUMBER CODE: 61130011 3A013001A91C -2

11 TITLE: (U) The Ultramicroscopic Structure of the Intestinal Mucosa in Tropical Sprue (PR)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical Medicine	12 64	"NA"	Other 1 DA

16 PROCURE METHOD	17 CONTRACT / GRANT a NUMBER / b TYPE / c DATE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b [illegible]
C	"NA"	PRIOR FY 65	1	15
		CURRENT FY 66	1	25

19 GOVT LAB INSTALLATION ACTIVITY
NAME / ADDRESS: Hqs US Army Medical Research & Dev Comd, Washington, D. C. 20315
RESP INDIV: Dacquisto, M. P., Lt Col
TEL: Ft Brooke PR 722-0000 Ext. 2101

20 PERFORMING ORGANIZATION
NAME / ADDRESS: U. S. Army Tropical Research Med. Lab., AFO New York 09851
INVESTIGATORS PRINCIPAL ASSOCIATE: Swanson, V. L., Dr.; Otero, L. R., Dr.
TEL: Ft Brooke PR 722-0000 Ext. 4147 TYPE: DA

21 TECHNOLOGY UTILIZATION	22 COORDINATION
"NA"	"NA"

23 KEYWORDS: Electron Microscope, Jejunal Biopsy, Tropical Sprue

24 (U) Tech Objective - Exploration at electron microscope magnifications of jejunal biopsy material from patients with tropical sprue constitutes the primary overall objective. Elucidation of the morphologic expressions of abnormal intestinal cell function is the technical objective, if not the actual visualization of an etiologic agent. In comparing normal ultra-structure of intestinal epithelial cells with the ultra-structure of diseased malabsorbing cells in tropical sprue, insight may be gained 25 incidentally into which subcellular structures are involved in normal absorption. Should a possible etiologic agent be seen, it would clearly narrow the scope of investigations now underway that are directed toward illuminating the pathogenesis of this condition.

(U) Approach - Selection of biopsy material for study has been based on extensive background information of the developmental stages of jejunal abnormality, worked out with the conventional light microscope. The rigid criteria set up for collection of material are: two untreated cases at each of five distinct and morphologically definable stages of abnormality, plus serial follow-up biopsies in treated cases which have 26 been selected for their typicallity, both clinically and pathologically.

(U) Progress - The reporting interval covered extends from the installation date of the electron microscope, 15 December 1964 to June 1965. A total of 38 specimens have been selected, processed and are ready for study. Sixteen of these fulfill all criteria outlined in the protocol of the project; the remainder will supply ancillary, corroborative information. Preliminary examination of some of the tissues discloses that definite abnormalities are present, but further study is required before firm conclusions can be drawn.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED [ ] b NOT RELATED [X]		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
"NA"	"NA"

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT
35 EST FUNDS (In thousands) FY [illegible]	36

DD FORM 1498A (Items 1 to 26 identical to NASA Form 1122)

ANNUAL PROGRESS REPORT

Project No. 3A013001A91C: In-House Laboratory Independent Research

Task No. 3A013001A91C: In-House Laboratory Independent Research

Work Unit No. A91C-2: The Ultramicroscopic Structure of the Intestinal Mucosa in Tropical Sprue (PR)

Reporting Installation: U. S. Army Tropical Research Medical Laboratory
APO New York 09851

Division: Pathology

Period Covered by Report: 1 July 1964 - 30 June 1965

Professional Authors:

Pathology Division: Virginia Swanson, MD

Univ. of Puerto Rico: L. Otero, PhD

Technical Assistants: Carl C. Ackerman, M/Sgt; Edna E. Nieves, BS.

Reports Control Symbol: RCS-MEDDH-288

Security Classification: UNCLASSIFIED

BODY OF REPORT

Project No. 3A013001A91C: In-House Laboratory Independent Research

Task No. 3A013001A91C: In-House Laboratory Independent Research

Work Unit No. A91C-2: The Ultramicroscopic Structure of the Intestinal Mucosa in Tropical Sprue (PR)

Progress:

The electron microscopic study of jejunal biopsy specimens from patients with tropical sprue, as indicated on subject DD 1498A, has progressed in a highly satisfactory manner. Observations made to date indicate: thickening of the epithelial basement membrane, the presence of isolated dense cells interspersed among normal columnar cells, disruption of the terminal web with adjacent unidentified dense bodies and multi-lobulation of nuclei with chromatic granules concentrated at the periphery.

Dr. Swanson, Principal Investigator, has unfortunately completed her overseas contract and will soon transfer to Armed Forces Institute of Pathology, where she plans to complete the morphological study of the tissue. Dr. Otero, University of Puerto Rico, has applied for an R&D contract in order to instigate further studies in intestinal absorption utilizing the Electron Microscope with emphasis on biochemical aberrations at the cellular level.

Summary:

Electron microscopic study of jejunal biopsy specimens from patients with tropical sprue is aimed towards elucidation of morphologic abnormalities that may be related to intestinal cell malfunction and the effort has been directed toward illuminating the pathogenesis of tropical sprue, possible causative factors and/or etiologic agents. The transfer of the principal investigator, Dr. Swanson, will result in the termination of this work unit.

RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYM
			DA 0A6409	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
1 07 65	A. New	RD U WRK U	"NA"	NL	A. Work Unit

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001A91C 01 085	61130011 3A013001A91C -3

11 TITLE (U) Immunologic Significance of Cross-Protection in Members of Group A Arboviruses (PR)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
010100 Microbiology	06 63	"NA"	Other | DA

16 PROCURE METHOD	17 CONTRACT GRANT		18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
	a NUMBER	a DATE			
C	"NA"		PRIOR FY 65	1	20
	c TYPE	d AMOUNT	CURRENT FY 66	1	20

19 GOVT LAB INSTALLATION ACTIVITY

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20 PERFORMING ORGANIZATION

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INVESTIGATORS PRINCIPAL ASSOCIATE Colon, J., Dr.
TEL Ft Brooke PR 722-0000 Ext. 4257 TYPE DA

21 TECHNOLOGY UTILIZATION	22 COORDINATION
"NA"	"NA"

23 KEYWORDS

Equine Encephalitis Viruses, Arboviruses, Group A Viruses, Gamma Globulin

24 (U) Tech Objective - The principal objective of this project is designed to study the development of the infectious particles of the equine encephalitis viruses, and the subordinate objective is to study the cross-protection among members of the arboviruses. These studies should enhance the understanding of the nature of the arboviruses, and help in the control of epidemics, control of pathogenicity, and production of vaccine.

25 (U) Approach - To work with isotopically labeled members of the group A viruses: Eastern, Venezuelan and Western equine encephalitis viruses. By means of that technic demonstrate that the immature forms that occur in the infected tissues are virus precursors and not decay products of mature virus (Colon, 1963). Purify the immature forms and study their relationship to the artificially formed infectious principles (Colon, 1963). Study the relationship between the core of the virus and the immature forms. By means of immunologic studies with purified gamma globulin demonstrate the antigenic relationship among the immature forms, core of the virus and other viral proteins. The major technical problem is the separation of the immature form of the virus from the mature form. One of the factors that may tend to accelerate or decelerate the work is the amount of purified material obtained from the preparation.

26 (U) Progress - We are conducting a serum survey to determine which are the arboviruses endemic in this area. This has to be done to avoid the introduction of a member of this group of viruses that is not present in the island.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED [X] b NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
"NA"	"NA"

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT
35 EST FUNDS (In thousands) FY	36

DD FORM 1498A (Items 1 to 26 identical to NASA Form 1112)

ANNUAL PROGRESS REPORT

Project No. 3A013001A91C:	In-House Laboratory Independent Research
Task No. 3A013001A91C:	In-House Laboratory Independent Research
Work Unit No. A91C-3:	Immunologic Significance of Cross-Protection in Members of Group A Arboviruses (PR)
Reporting Installation:	U. S. Army Tropical Research Medical Laboratory APO New York 09851
Division:	Microbiology
Period Covered by Report:	1 July 1964 - 30 June 1965
Professional Author:	Julio Colon, PhD
Technical Assistants:	John F. Altman, SFC; Roland Lewis, S/Sgt; Rogelio R. Garibay, SP6; Victor A. Lopez; Edna E. Nieves, BS.
Reports Control Symbol:	RCS-MEDDH-288
Security Classification:	UNCLASSIFIED

BODY OF REPORT

Project No. 3A013001A91C: In-House Laboratory Independent Research

Task No. 3A013001A91C: In-House Laboratory Independent Research

Work Unit No. A91C-3: Immunologic Significance of Cross-Protection in Members of Group A Arboviruses (PR)

Progress:

A limited survey has been carried out for the presence of hemagglutination-inhibiting antibodies against a number of arthropod-borne viruses. The sera were collected in this laboratory from healthy residents from different regions of Puerto Rico.

Wild animals (rats, mongooses, bats, etc.) have been captured and some of their organs have been cultured for the presence of arboviruses. Serums of wild animals are also being tested for the presence of antibodies against some of the arboviruses.

Summary:

A serum survey is being conducted to determine which are the arboviruses endemic in Puerto Rico. A search for arboviruses is being conducted in wild animals in Puerto Rico.

APPENDIX

TABLE 1

Types of Enteroviruses Isolated in Cantera from December 1963 to March 1965 from Children with Diarrhea

Month	Virus Type	Number of Cases	Total
December	Echo 20	1	
	Coxsackie A-9	1	2
Dec - January	Echo 8*	7	7
January	Echo 7*	1	
	Echo 15	2	
	Echo 16	2	5
April	Echo 11*	3	3
April - May	Echo 3	3	3
May	Echo 14	1	
	Echo 17	3	4
June	Coxsackie B-6	2	2
July	Echo 6	1	
	Echo 18	1	
	Polio III**	1	3
July, April, February	Polio II**	4	4

* These viruses were responsible for two big winter outbreaks, Echo 8 in Ponce and Echo 11 and Echo 7 in Cidra.

** Children were vaccinated with Sabin Vaccine at the time.

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TABLE 2

Age Distribution of Viral Antibodies in Puerto Rican Children*

Age Group	Total No. of Patients	Viruses				
		Echo 3	Echo 7	Echo 8	Echo 9	Coxsackie A-9
≤6 months	147	21	42	17	13	13
6 months - <1 yr.	104	15	15	12	7	17
1 year - <3 years	302	86	113	61	37	68
3 - < 6 years	275	126	125	75	86	84
6 - < 9 years	53	40	39	29	24	21
9 - <12 years	34	26	18	15	12	10
12 years	75	49	10	33	37	33
Unknown	10	5	5	3	4	4
TOTAL	1000	368	367	245	220	250
Percentage		36.8	36.7	24.5	22.0	25.0

Six different areas of Puerto Rico were included:

Guaynabo, Barrio Obrero, Puerta de Tierra, Manzanilla, Cialitos, and San Juan Metropolitan Area.

TABLE 3

Percentage of Children with Serum Neutralizing Antibodies Against Enteric Viruses in Some Areas of Puerto Rico*

Areas of Puerto Rico	No. Patients	Viruses				
		Echo 3 %	Echo 7 %	Echo 8 %	Echo 9 %	Coxsackie A-9 %
Guaynabo	84	47.6	47.6	39.2	13	22.6
Barrio Obrero	148	43.9	51.3	22.9	19.6	13.5
Puerta de Tierra	290	50.7	51.2	25.8	34.5	39.6
Manzanilla	87	24.1	43.6	55.1	24.1	34.4
Cialitos	175	20.0	20.0	18.8	18.8	17.7

*Age group was from 1 month to 12 years.

TABLE 4

Percentage of Individuals of Given Age with Serum Antibodies Against Indicated Enteric Viruses*

Age Group	VIRUSES				
	Echo 3 %	Echo 7 %	Echo 8 %	Echo 9 %	Coxsackie A-9
< 6 months	15.3	28.7	10.7	8.7	8.7
6 months - <1 yr.	13.0	13.9	12.0	7.4	14.8
1 - <3 years	28.5	37.7	20.6	12.1	22.3
3 - <6 years	46.0	44.4	27.4	31.8	31.4
6 - <9 years	80.0	80.0	56.0	48.0	42.0
9 - 12 years	92.6	59.3	51.9	44.4	37.0
<12 years	78.4	80.4	60.8	66.7	51.0

* Data collected from six different areas of Puerto Rico.

TABLE 5

Specificity of the Antibodies Present in Human Colostrum

Sample	Before Absorption EPEC Type - Titer		Absorbed With	After Absorption EPEC Type - Titer		Titer Drop
1 CBS*	0126:B16 055:B5	1:32 1:64	0126:B6	0126:B16 055:B5	1:16 1:64	2X 0
3 C_3	0111:B4 0126:B16	1:1024 1:512	0111:B4	0111:B4 0126:B16	1:8 1:512	512X 0
7 MBS**	0126:B16 0119:B14	1:32 1:64	0126:B6	0126:B16 0119:B14	1:16 1:32	2X 2X
8 C_1***	055:B5 0126:B16	1:512 1:1024	055:B5	055:B5 0126:B16	1:8 1:1024	64X 0
10 C_2	055:B5 0119:B14	1:512	055:B5	055:B5 0119:B14	1:16 1:1024	32X 0
10 C_9	055:B5 0119:B14	1:512 1:512	055:B5	055:B5 0119:B14	1:16 1:512	32X 0
21 C_3	0119:B14 055:B5	1:256 1:1024	0119:B14	0119:B14 055:B5	1:8 1:1024	32X 0

*CBS = cord blood serum
**MBS = mother blood serum
***C = colostrum

TABLE 6

Family	Patient	Relation	Age in Years	Duration of Symptoms	Isolation Dates	Serum Dates	HA Titers
3	1	Sib	2 ½	10 to 22 Oct	15 Oct 22 Oct	23 Sept 29 Dec	32 128
	2	Mother	20	6 to 8 July	25 Jun 1 Jul	19 Mar 24 Jun 23 Sep	0 8 16
	5	Father	24	29 Jun and 6 Jul	7 Jul	19 Mar 7 Jul 25 Sep	0 8 16
	6	Aunt*	12	asymptom	22 Sep	24 Jun 23 Sep 21 Jan	0 0 128
	7	Triplet Sib	7/12	27 to 29 Oct	27 Oct	23 Sep 16 Dec	0 128
	8	Triplet Sib	7/12	21 Oct to 6 Nov	27 Oct 28 Oct	23 Oct 16 Dec	0 256
	9	Triplet Sib	7/12	26 Oct to 1 Nov	26 Oct 27 Oct 29 Oct	23 Sep 16 Dec	0 512
9	1	Sib	5	26 - 27 Oct	27 Oct 28 Oct	22 Sep 16 Dec	32 128
	3	Sib	1 5/12	14 - 21 Dec	14 Dec 30 Dec	22 Sep 30 Dec	0 1024

*Baby Sitter

TABLE 7

Enteropathogens Recovered from Children
in Cidra Health Center

Microorganisms	Number Isolated	Percent
EPEC	8	20
Shigellae	2	5
Enteric Viruses	17	49

*Number of patients with diarrhea for Bact. = 40
Number of patients with diarrhea for Virology = 35

TABLE 8

Viruses Recovered from Children
in Cidra Health Center

Viruses	Number Isolated	Percent
Echo 11	8	47
Echo 7	5	30
Polioviruses	2	12
Coxsackie B-5	1	6
Unknown	1	6

TABLE 9

Enteropathogens Isolated in CPD Patients in Cidra

Enteropathogens	Number Isolated	Percent
EPEC	6	24
Shigella	2	7
Enteroviruses	12	45
Echo 11 virus	7	
Echo 7 virus	1	
Poliovirus	1	
Others	2	

TABLE 10

Enteropathogens Isolated in Hospitalized Patients in Cidra

Enteropathogens	Number Isolated	Percent
EPEC	2	18
Enteroviruses	5	63
Echo 7 viruses	4	
Echo 11 virus	1	

TABLE 11

Increase in Neutralizing Antibodies for Echo 7 and Echo 11 Viruses in Cidra Patients

Virus	Number with Increase*	Percent
Echo 7	17	85
Echo 11	20	100
Echo 7 and Echo 11	17	85

*An increase in titer of 8 folds or more was taken as positive.

TABLE 12

Duration of Diarrhea in Patients that Showed Rise in Neutralizing Antibody Titer to Echo 7 and Echo 11 Viruses

Patient	Age	Days with Diarrhea
1-1	5 months	2
1-2*	4 years	2
1-3*	7 years	21
1-6	1.5 years	30
1-11	7 months	19
1-12	2 years	4
1-13	1 year	10
1-14	9 years	3
1-20*	3 years	14
1-21	9 months	23
1-22	36 days	19
1-25**	1 year	10
2-10**	4 years	4
2-11*	3 years	15
2-14	6 months	21
2-15*	5 years	2
2-17*	6 years	8

*Showed experience with E. coli 055:B5

**Showed rise in hemagglutinating titer to E. coli 055:B5.

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY *(Corporate author)*
U. S. Army Tropical Research Medical Laboratory
APO New York 09851

2a. REPORT SECURITY CLASSIFICATION
UNCLASSIFIED

2b. GROUP

3. REPORT TITLE
Annual Progress Report FY 1965

4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*
Annual Report 1 July 1964 - 30 June 1965

5. AUTHOR(S) *(Last name, first name, initial)*
Frick, Lyman P. Colonel, MSC

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
30 September 1965	115	

8a. CONTRACT OR GRANT NO.

b. PROJECT NO.

c.

d.

9a. ORIGINATOR'S REPORT NUMBER(S)

9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)*
RCS-MEDDH-288

10. AVAILABILITY/LIMITATION NOTICES
Qualified requestors may obtain copies of this report from DDC.

11. SUPPLEMENTARY NOTES

12. SPONSORING MILITARY ACTIVITY
U. S. Army Medical Research and Development Command, Office of The Surgeon General, Department of the Army
Washington, D. C. 20315

13. ABSTRACT

The research program of the U. S. Army Tropical Research Medical Laboratory is devoted to the study of tropical diseases of military importance.

The research projects described herein are related to investigations of Schistosomiasis, Tropical Sprue and Common Diarrheas.

DD FORM 1 JAN 64 1473

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Annual progress report, military internal medicine, communicable diseases, in-house laboratory independent research, immunology, metabolism, nutrition, zoological sciences, diarrheal diseases, parasitic diseases, human chromosomes, tropical sprue, arboviruses, echoviruses, schistosomiasis, gastrointestinal function, electron microscope, biopsy capsule, enzyme, mucosa						

INSTRUCTIONS

1. ORIGINATING ACTIVITY: Enter the name and address of the contractor, subcontractor, grantee, Department of Defense activity or other organization *(corporate author)* issuing the report.

2*a*. REPORT SECURITY CLASSIFICATION: Enter the overall security classification of the report. Indicate whether "Restricted Data" is included. Marking is to be in accordance with appropriate security regulations.

2*b*. GROUP: Automatic downgrading is specified in DoD Directive 5200.10 and Armed Forces Industrial Manual. Enter the group number. Also, when applicable, show that optional markings have been used for Group 3 and Group 4 as authorized.

3. REPORT TITLE: Enter the complete report title in all capital letters. Titles in all cases should be unclassified. If a meaningful title cannot be selected without classification, show title classification in all capitals in parenthesis immediately following the title.

4. DESCRIPTIVE NOTES: If appropriate, enter the type of report, e.g., interim, progress, summary, annual, or final. Give the inclusive dates when a specific reporting period is covered.

5. AUTHOR(S): Enter the name(s) of author(s) as shown on or in the report. Enter last name, first name, middle initial. If military, show rank and branch of service. The name of the principal author is an absolute minimum requirement.

6. REPORT DATE: Enter the date of the report as day, month, year, or month, year. If more than one date appears on the report, use date of publication.

7*a*. TOTAL NUMBER OF PAGES: The total page count should follow normal pagination procedures, i.e., enter the number of pages containing information.

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8*a*. CONTRACT OR GRANT NUMBER: If appropriate, enter the applicable number of the contract or grant under which the report was written.

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